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ABSTRACT:

The present invention relates to insoluble compositions comprising a protein selected from the group consisting of insulin, insulin analogs, and proinsulins; a derivatized protein selected from the group consisting of derivatized insulin, derivatized insulin analog, and derivatized proinsulin; a complexing compound; a hexamer-stabilizing compound; and a divalent metal cation. Formulations of the insoluble composition are suitable for both parenteral and non-parenteral delivery for treating hyperglycemia and diabetes. Microcrystal forms of the insoluble precipitate are pharmaceutically analogous to the neutral protamine Hagedorn (NPH) insulin crystal form. Surprisingly, it has been discovered that suspension formulations of such insoluble compositions possess unique and controllable dissolution properties that provide therapeutically advantageous glucodynamics compared with insulin NPH formulations.

47 Claims, 4 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 4

Brief Summary Text - BSTX (10):

NPH insulin microcrystals possess a distinctive rod-shaped morphology of typical dimensions about 5 **microns** long by 1 **micron** thick and 1 **micron** wide. The extended duration of action of NPH insulin microcrystals results from their slow absorption from the subcutaneous injection site.

Detailed Description Text - DETX (6):

The term, "microcrystal" means a solid that is comprised primarily of matter in a crystalline state, wherein the individual crystals are predominantly of a single crystallographic composition and are of a microscopic size, typically of longest dimension within the range 1 **micron** to 100 **microns**. The term "microcrystalline" refers to the state of being a microcrystal.

Detailed Description Text - DETX (36):

An "isotonicity agent" is a compound that is physiologically tolerated a imparts a suitable tonicity to a formulation to prevent the net flow of water across cell **membranes** that are in contact with an administered formulation. Glycerol, which is also known as glycerin, is commonly used as an isotonicity agent. Other isotonicity agents include salts, e.g., sodium chloride, and monosaccharides, e.g., dextrose and lactose.

Detailed Description Text - DETX (43):

As mentioned above, the present invention provides insoluble compositions that have properties similar to NPH insulin in certain respects, and superior to NPH insulin in other respects. They are similar to NPH insulin in respect to their physical properties, as described hereafter. A light microscope equipped with an oil immersion objective and a crossed polarizer was utilized to examine microcrystals comprised of B29-N.epsilon.-octanoyl-human insulin, insulin, zinc, protamine, and phenol prepared according to the present invention. Examination at 1000x magnification showed that these microcrystals were single and rod-like, exhibiting a uniform crystal morphology. The sizes of these microcrystals fell generally within the range of approximately 2 **microns** long to 8 **microns** long. A direct comparison using this microscope showed that the morphology of these microcrystals appeared to be similar to that of commercially manufactured pork NPH microcrystals, which has elsewhere been described as rod-like. The size range of these microcrystals was also similar to that of commercially manufactured NPH microcrystals, which generally have an average length of about 5 **microns**. The commercial manufacturing specification for the mean length of NPH microcrystals is from 1 **micron** to 40 **microns**.

Detailed Description Text - DETX (48):

The dissolution rate of pork insulin NPH microcrystals was measured by placing 5 microliters of U100 pork insulin NPH into 3 mL of Dulbecco's phosphate buffered saline (without calcium or magnesium) in a 1 cm path length square quartz cuvette at a **temperature** of 22.degree. C. This solution was stirred at a constant rate using a magnetic cuvette stirrer. Absorbance measurements at 320 nm were taken at 1 minute intervals. The absorbance at 320 nm corresponds to the light scattered by the insoluble particles present in the aqueous suspension. Consequently, as the microcrystals dissolve, the absorbance approaches zero. Pork insulin NPH microcrystals were completely dissolved after about 20 minutes.

Detailed Description Text - DETX (51):

A procedure as described above was followed to measure the dissolution rate of these co-crystals. In summary, a volume of 12 microliters of each protamine-zinc-B29-

N.epsilon.-octanoyl-LysB29 human insulin-human insulin co-crystalline suspension (containing no more than 50 U/mL) was placed into 3 mL of Dulbecco's phosphate buffered saline (without calcium or magnesium) in a 1 cm path length square quartz cuvette. This solution was stirred at the same constant rate and at the same **temperature** of 22.degree. C. The data generated from this experiment are presented in FIG. 1, and show that the 3:1 co-crystals required more than 100 minutes to dissolve, that the 1:1 co-crystals required more than 150 minutes to dissolve, and that the 1:3 co-crystals required did not completely dissolve even after 400 minutes.

Detailed Description Text - DETX (121):

The microcrystals of the present invention are preferably oblong-shaped, also known as "rod-like", single crystals that are comprised of a protein, a derivatized protein, a divalent cation, and including a complexing compound and a hexamer-stabilizing compound. The mean length of the microcrystals of the present invention preferably is within the range of 1 **micron** to 40 **microns,** and more preferably is within the size range of 3 **microns** to 15 **microns**.

Detailed Description Text - DETX (135):

Crystallization parameters that influence the crystallization rate and the size of crystals of the present invention are: acyl group size and nature; **temperature**; the presence and concentration of compounds that compete with the protein and derivatized protein for zinc, such as citrate, phosphate, and the like; the nature and concentration of phenolic compound(s); zinc concentration; the presence and concentration of a miscible organic solvent; the time permitted for crystallization; the pH and ionic strength; buffer identity and concentration; the concentration of precipitants; the presence of seeding materials; the shape and material of the container; the stirring rate; and the total protein concentration. **Temperature** and the concentration of competing compounds are thought to be of particular importance.

Detailed Description Text - DETX (137):

An example of a process for preparing the precipitates and crystals of the present invention follows. A measured amount of the derivatized protein and a measured amount of the protein are dissolved in, or are combined to form a solution in an aqueous solvent containing a hexamer-stabilizing compound, such as a phenolic compound. To this solution is added a solution of zinc as one of its soluble salts, for example Zn(II)Cl.sub.2, to provide from about 0.3 moles of zinc per mole of derivatized insulin to about 0.7 moles, or to as much as 1.0 moles, of zinc per mole of total protein (protein+derivatized protein). Absolute ethanol, or another miscible organic solvent, may optionally be added to this solution in an amount to make the solution from about 5% to about 10% by volume organic solvent. This solution may then be filtered through a 0.22 **micron,** low-

protein binding filter. A protamine solution is prepared by dissolving a measured amount of protamine in an aqueous solvent. This solution may be filtered through a 0.22 **micron,** low-protein binding filter. The solution of protein and derivatized protein and the protamine solution are combined, whereupon a precipitate forms initially. The resulting suspension is stirred slowly at room **temperature** (typically about 20-25.degree. C.), whereupon microcrystals are formed within a period from about 4 hours to about 10 days.

Detailed Description Text - DETX (139):

In another process for preparing the insoluble compositions of the present invention, for example, a measured mass of dry derivatized protein and a measured mass of dry protein are dissolved together in an acidic aqueous solvent, such as 0.1 N-1.0 N HCl. This solution is stirred to insure thorough mixing of derivatized protein and protein. The ratio of derivatized protein powder to protein powder in this mixture is predefined to achieve a similar ratio of derivatized protein to protein in the insoluble composition to be produced. A separately prepared aqueous solution comprised of a phenolic preservative and, optionally, a pharmaceutically acceptable buffer, is combined with the acidic solution of the proteins. The pH of the resulting solution is then adjusted to about 6.8 to about 8.4, preferably from about 6.8 to about 8.0, or preferably to a pH of from about 7.2 to about 7.8, and most preferably from about 7.4 to about 7.8. To this solution is added a solution of zinc as one of its soluble salts, for example Zn(II)Cl.sub.21 to provide from about 0.3 moles of zinc per mole of total insulin to about 4 moles of zinc per mole of total insulin. This solution is adjusted to a pH as given above, and preferably to about 7.4-7.6, and may then be filtered through a 0.22 **micron,** low-protein binding filter. A solution of protamine is prepared by dissolving a measured mass of protamine in an aqueous solvent. The protamine solution may be filtered through a 0.22 **micron,** low-protein binding filter. The solution of protein and derivatized protein and the protamine solution are combined, whereupon a precipitate forms initially. The resulting suspension is stirred slowly at room **temperature** (typically about 20-25.degree. C.), whereupon microcrystals are formed within a period from about 4 hours to about 10 days.

Detailed Description Text - DETX (140):

In another process for preparing the insoluble compositions of the present invention, a measured amount of a derivatized protein is first dissolved in an aqueous solvent containing a phenolic preservative. To this solution is added a solution of zinc as one of its soluble salts, for example Zn(II)Cl.sub.2, to provide from about 0.3 moles of zinc per mole of derivatized protein to about 4 moles of zinc per mole of derivatized protein. The pH of the resulting solution is then adjusted to about 6.8 to about 8.4, preferably from about 6.8 to about 8.0, or preferably to a pH of from about 7.2 to about 7.8, and most preferably from about 7.4 to about 7.8. A second solution is prepared separately wherein

a measured amount of a protein selected from the group consisting of insulin, insulin analogs, and proinsulin is dissolved in an aqueous solvent containing a phenolic preservative. To this solution is added a solution of zinc as one of its soluble salts, for example Zn(II)Cl.sub.2, to provide from about 0.3 moles of zinc per mole of protein to about 4 moles of zinc per mole of protein. The pH of the resulting solution is then adjusted to about 6.8 to about 8.4, preferably from about 6.8 to about 8.0, or preferably to a pH of from about 7.2 to about 7.8, and most preferably from about 7.4 to about 7.8, or 7.4-7.6. Portions of the derivatized protein solution and the protein solution are combined in a ratio that is predefined in order to achieve a similar ratio of derivatized protein to protein in the insoluble composition. This solution is stirred to insure thorough mixing of derivatized protein and protein. This solution is then adjusted to a pH of about 7.6, and may then be filtered through a 0.22 **micron,** low-protein binding filter. A protamine solution is prepared separately by dissolving a measured amount of protamine in an aqueous solvent. This protamine solution may be filtered through a 0.22 **micron,** low-protein binding filter. The solution of protein and derivatized protein and the protamine solution are combined, whereupon a precipitate forms initially. The resulting suspension is stirred slowly at room **temperature** (typically about 20-25.degree. C.), whereupon microcrystals are formed within a period from about 4 hours to about 10 days.

Detailed Description Text - DETX (145):

The following preparations and examples illustrate and explain the invention. The scope of the invention is not limited to these preparations and examples. Reference to "parts" for solids means parts by weight. Reference to "parts" for liquids means parts by volume. Percentages, when used to express concentration, mean mass per volume (.times.100). All **temperatures** are degrees Centigrade (.degree. C.). "TRISO refers to 2-amino-2-hydroxymethyl-1,3,-propanediol. The 1000 part-per-million (ppm) zinc solution was prepared by diluting 1.00 mL of a 10,000 ppm zinc atomic absorption standard solution [Ricca Chemical Company, zinc in dilute nitric acid] with water to a final volume of 10.00 mL.

Detailed Description Text - DETX (150):

In many of the preparations described below, a standard spectrophotometric assay was used to determine how rapidly the crystals dissolved in Dulbecco's phosphate buffered saline (pH 7.4) at room **temperature**. Significant deviations from the procedure described immediately below are noted where appropriate in the descriptions of the preparations. A spectrophotometer suitable for measuring in the ultraviolet range, and equipped with a 1 cm cuvette and a magnetic cuvette stirrer was used for all the dissolution assays. The cuvette, containing a small stir bar and 3.00 mL of phosphate buffered saline (PBS), was put into the cell compartment of the spectrophotometer. The instrument was set to 320 nm and zeroed against the same buffer. Then 4.0 microliters of

a well suspended formulation, usually having a total concentration approximately equivalent to a U50 formulation, or about 1.6 to 1.8 mg/mL, was added to the cuvette. After waiting 1.0 minute for mixing, the optical density at 320 nm was recorded. Since the proteins involved in this work do not absorb light at 320 nm, the decrease in optical density was due to reduction in light scattering as the crystals dissolved. The time for the optical density to drop to half of its initial value is typically reported (t1/2). As a control, 2.0 microliters of U100 Humulin.RTM. N (i.e., human insulin NPH, which is also known as human NPH density at 320 nm monitored as above. The dissolution half-time (t1/2) for the Humulin.RTM. N formulation was about 6 minutes.

Detailed Description Text - DETX (153):

A dry powder of B29-N.epsilon.-octanoyl-LysB29 human insulin (0.7 parts by mass) and a dry powder of human insulin (6.3 parts by mass) are dissolved in 1000 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6. To this solution is added 75 parts of a 15.3 mM solution of zinc chloride. The pH is adjusted to 7.6 with 1 N HCl and/or 1 N NaOH. This solution is filtered through a 0.22 **micron,** low-protein binding filter. A second solution is prepared by dissolving 7 parts by mass of protamine sulfate in 10,000 parts by volume of water then filtering through a 0.22 **micron,** low-protein binding filter. Equal volumes of the solution containing insulin and acylated insulin and of the protamine sulfate solution are combined. Initially, an amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate converts to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (156):

The procedure of Preparation 1 is followed, except that 1.75 parts by mass of a dry powder of B29-N.epsilon.-octanoyl-LysB29 human insulin and 5.25 parts by mass of a dry powder of human insulin are used. After equal volumes of the solution containing insulin and acylated insulin and of the protamine sulfate solution are combined, an amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate converts to a co-crystalline microstalline solid.

Detailed Description Text - DETX (159):

The co-crystalline microcrystals prepared by the method of Preparation 1 are separated from the supernatant and are recovered by conventional solid/liquid separation methods, such as, **filtration,** centrifugation, or decantation. The recovered co-crystalline microcrystals are then suspended in a solution consisting of 25 mM TRIS, 5 mg/ml phenol, and 16 mg/ml glycerol, pH 7.8, so that the final concentration of insulin activity is about 100 U/mL.

Detailed Description Text - DETX (162):

The procedure of Preparation 1 is followed, except that 3.5 parts by mass of a dry powder of B29-N.epsilon.-octanoyl-LysB29 human insulin and 3.5 parts by mass of a dry powder of human insulin are used. After equal volumes of the solution containing insulin and acylated insulin and of the protamine sulfate solution are combined, an amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate converts to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (165):

The procedure of Preparation 1 is followed, except that 5.25 parts by, mass of a dry powder of B29-N.epsilon.-octanoyl-LysB29 human insulin and 1.75 parts by mass of a dry powder of human insulin are used. After equal volumes of the solution containing insulin and acylated insulin and of the protamine sulfate solution are combined, an amorphous recipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate converts to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (168):

The procedure of Preparation 1 is followed, except that 1.75 parts by mass of a dry powder of B29-N.epsilon.-hexanoyl-LysB29 human insulin and 5.25 parts by mass of a dry powder of human insulin are used. After equal volumes of the solution containing insulin and acylated insulin and of the protamine sulfate solution are combined, an amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate will convert to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (171):

The procedure of Preparation 1 is followed, except that 1.75 parts by mass of a dry powder of B29-N.epsilon.-butyryl-LysB29 human insulin and 5.25 parts by mass of a dry powder of human insulin are used. After equal volumes of the solution containing insulin and acylated insulin and of the protamine sulfate solution are combined, an amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate will convert to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (175):

To each of these five solutions, 1.6 mL of a solvent composed of 50 mM TRIS buffer, 0.1 M trisodium citrate, and 10 mg/mL phenol at pH 7.6 was added. To each of the five solutions, 0.15 ml of a 15.3 mM solution of zinc chloride was added. Each of the resulting five solutions were adjusted to a pH of 7.6 with 1 N NaOH. Each of the resulting five solutions were filtered through a 0.22 **micron,** low-protein binding filter. An additional solution was prepared by dissolving 3.50 mg of protamine sulfate in 10 mL of water then filtered through a 0.22 **micron,** low-protein binding filter. A volume of 1.9 mL of each of the five solutions and 1.9 mL of the protamine sulfate solution were combined respectively, in each of the five solutions resulting in the immediate appearance of an amorphous precipitate. These five solutions were allowed to stand for 24 hours at room **temperature** (approximately 22.degree. C.). This procedure resulted in the formation of a white-to-off-white microcrystalline solid in each of the five solutions.

Detailed Description Text - DETX (178):

A dry powder of B29-N.epsilon.-octanoyl-LysB29 human insulin (0.7 parts by mass) is dissolved in 100 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6. To this solution is added 7.5 parts of a 15.3 mM solution of zinc chloride. A second solution is prepared wherein a dry powder of human insulin (6.3 parts by mass) is dissolved in 900 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6. To this solution is added 67.5 parts of a 15.3 mM solution of zinc chloride. The acylated insulin solution and the insulin solution are combined together and stirred to insure mixing of the two solutions. This solution is filtered through a 0.22 **micron,** low-protein binding filter. A protamine solution is prepared by dissolving 7 parts by mass of protamine sulfate in 10,000 parts by volume of water then filtering through a 0.22 **micron,** low-protein binding filter. Equal volumes of the acylated insulin solution and of the protamine sulfate solution are combined. An amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate will convert to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (181):

A dry powder of B29-N.epsilon.-octanoyl-LysB29 human insulin. (1.75 parts by mass) is dissolved in 250 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6. To this solution is added 18.75 parts of a 15.3 mM solution of zinc chloride. A second solution is prepared wherein a dry powder of human insulin (5.25 parts by mass) is dissolved in 750 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6., To this solution is added 56.25 parts of a 15.3 mM solution of zinc chloride. The acylated insulin solution and the insulin solution are combined together and

stirred to insure mixing of the two solutions. This solution is filtered through a 0.22 **micron,** low-protein binding filter. A protamine solution is prepared by dissolving 7 parts by mass of protamine sulfate in 10,000 parts by volume of water then filtering through a 0.22 **micron,** low-protein binding filter. Equal volumes of the acylated insulin solution and of the protamine sulfate solution are combined. An amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate converts to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (184):

A dry powder of B29-N.epsilon.-octanoyl-LysB29 human insulin (3.5 parts by mass) is dissolved in 500 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6. To this solution is added 1.75 parts of a 15.3 mM solution of zinc chloride. A second solution is prepared wherein a dry powder of human insulin (3.5 parts by mass) is dissolved in 500 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6. To this solution is added 37.5 parts of a 15.3 mM solution of zinc chloride. The acylated insulin solution and the insulin solution are combined together and stirred to insure mixing of the two solutions. This solution is filtered through a 0.22 **micron,** low-protein binding filter. A protamine solution is prepared by dissolving 7 parts by mass of protamine sulfate in 10,000 parts by volume of water then filtering through a 0.22 **micron,** low-protein binding filter. Equal volumes of the acylated insulin solution and of the protamine sulfate solution are combined. An amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate converts to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (187):

A dry powder of B29-N.epsilon.-octanoyl-LysB29 human insulin (5.25 parts by mass) is dissolved in 750 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6. To this solution is added 56.25 parts of a 15.3 mM solution of zinc chloride. A second solution is prepared wherein a dry powder of human insulin (1.75 parts by mass) is dissolved in 250 parts by volume of an aqueous solvent composed of 50 mM TRIS, 0.1 M trisodium citrate, and 10 mg/ml phenol at pH 7.6. To this solution is added 18.75 parts of a 15.3 mM solution of zinc chloride. The acylated insulin solution and the insulin solution are combined together and stirred to insure mixing of the two solutions. This solution is filtered through a 0.22 **micron,** low-protein binding filter. A protamine solution is prepared by dissolving 7 parts by mass of protamine sulfate in 10,000 parts by volume of water then filtering through a 0.22 **micron,** low-protein binding filter. Equal volumes of the acylated insulin

solution and of the protamine sulfate solution are combined. An amorphous precipitate forms. This suspension is allowed to stand for about 24 hours at room **temperature** (typically about 22.degree. C.). The amorphous precipitate converts to a co-crystalline microcrystalline solid.

Detailed Description Text - DETX (190):

An acidic solution of B29-N.epsilon.-hexanoyl-human insulin was prepared by dissolving 12.3 mg of B29-N.epsilon.-hexanoyl-human insulin in 0.3 mL of 0.1 N HCl. An acidic solution of human insulin was prepared by dissolving 4.6 mg of human insulin (zinc crystals) in 0.1 mL of 0.1 N HCl. The two solutions were combined giving a total volume of 0.4 mL. This resulting solution was stirred for approximately 5 minutes. To this resulting solution was added, with stirring, 0.150 mL of a 1000 ppm zinc(II) solution. A crystallization diluent was prepared comprising 32 mg/mL glycerol, 50 mM tris buffer, 10 mg/mL phenol, 100 mM trisodium citrate, at a pH of 7.6. To the insulin solution was added 1.6 mL of the crystallization diluent. The pH of the solution was adjusted to 7.59 using 1 N NaOH and 1 N HCl. The solution was filtered through a 0.22 **micron,** low protein binding filter. A protamine solution was prepared by dissolving 7.47 mg of protamine sulfate in 10 mL of water. Two milliliters (2 mL) of the protamine solution was added to 2 mL of the insulin solution. The resulting solution was allowed to stand undisturbed for 18 hours at a controlled **temperature** of 25.degree. C.

Detailed Description Text - DETX (191):

Microscopic inspection (at 18 hours) revealed that crystallization had occurred and that the preparation yielded uniform, single, rod-like crystals possessing approximate average lengths of 3 **microns**.

Detailed Description Text - DETX (193):

The dissolution rate of the crystals was measured by placing 0.005 mL of the uniformly suspended formulation into 3 mL of Dulbecco's phosphate buffered saline (without calcium or magnesium) in a 1 cm path length square quartz cuvette at a **temperature** of 22.degree. C. This solution was stirred at a constant rate using a magnetic cuvette stirrer. Absorbance measurements at 320 nm were taken at 1 minute intervals. The absorbance at 320 nm corresponds to the light scattered by the insoluble particles present in the aqueous suspension. Consequently, as the microcrystals dissolve, the absorbance approaches zero. The time required for the 0.005 mL of this formulation to dissolve was greater than 150 minutes. The time required for dissolution of a 0.005 mL sample of U100 commercial Humulin N to dissolve when subjected to the same conditions was about 10 minutes.

Detailed Description Text - DETX (200):

An acidic solution of B29-N.epsilon.-decanoyl-human insulin was prepared by dissolving 10.4 mg of B29-N.epsilon.-decanoyl-human insulin in 0.25 mL of 0.1 N HCl. An acidic solution of human insulin was prepared by dissolving 30.3 mg of human insulin (zinc crystals) in 0.75 mL of 0.1 N HCl. The two solutions were combined, giving a total volume of 1 mL. This resulting solution was stirred for approximately 5 minutes. To this solution was added, with stirring, 0.305 mL of a 1000 ppm zinc(II) solution. To the resulting solution, was added 4 mL of a crystallization diluent (40 mg/mL glycerol, 50 mM tris buffer, 4 mg/mL m-cresol, 1.625 mg/mL phenol, 100 mM trisodium citrate, pH 7.4). The pH of the resulting solution was adjusted to 7.58. This solution was filtered through a 0.22 **micron,** low protein binding filter. Five milliliters (5 mL) of protamine solution (37.6 mg of protamine sulfate in 50 mL of water) was added to 5 mL of the filtered solution. The resulting solution was allowed to stand undisturbed for 63 hours at a controlled **temperature** of 25.degree. C.

Detailed Description Text - DETX (201):

Microscopic inspection (at 63 hours) revealed that crystallization had occurred, and that the preparation had yielded uniform, single, rod-like crystals possessing approximate average lengths of 8 **microns**.

Detailed Description Text - DETX (202):

The dissolution rate of the crystals was measured by placing 0.006 mL of the uniformly suspended crystal formulation into 3 mL of Dulbecco's phosphate buffered saline (without calcium or magnesium) in a 1 cm path length square quartz cuvette at a **temperature** of 22.degree. C. The time required for the 0.006 mL of this crystal formulation to dissolve was greater than 300 minutes. The time required for a 0.005 mL sample of U100 commercial Humulin N to dissolve under the same conditions was about 10 minutes.

Detailed Description Text - DETX (205):

A particle sizing measurement was performed on a sample of the formulation utilizing a particle sizing instrument (Multisizer Model IIE, Coulter Corp., Miami, Fla. 33116-9015). To perform this measurement, 0.25 mL of the crystal formulation was added to 100 mL of a diluent consisting 14 mM dibasic sodium phosphate, 16 mM glycerol, 1.6 mg/mL m-cresol, 0.65 mg/mL phenol, pH 7.4. The instrument aperture tube orifice size was 50 **microns**. Particle size data was collected for 50 seconds. This measurement showed that the mean particle diameter of the crystals was approximately 6 **microns** with an approximately normal distribution encompassing a range of particle sizes from approximately 2 **microns** to approximately 9 **microns**. This result is similar to the particle size distribution of commercial NPH determined using an analogous method [DeFelippis, M. R., et al. J. Pharmaceut. Sci. 87:170-176 (1998)].

Detailed Description Text - DETX (208):

An acidic solution of B29-N.epsilon.-octanoyl-human insulin was prepared by dissolving 30.3 mg of B29-N.epsilon.- octanoyl-human insulin in 0.75 mL of 0.1 N HCl. An acidic solution of human insulin was prepared by dissolving 59.7 mg of human insulin (zinc crystals) in 1.5 mL of 0.1 N HCl. An aliquot (0.25 mL) of the human insulin solution was combined with the 0.75 mL B29-N.epsilon.-octanoyl-human insulin solution, giving a total volume of 1 mL, which was stirred for approximately 5 minutes. To this was added, with stirring, 0.365 mL of a 1000 ppm zinc(II) solution. To the insulin plus zinc solution was added 4 mL of crystallization diluent (40 mg/mL glycerol, 35 mM sodium phosphate dibasic buffer, 4 mg/mL m-cresol, 1.625 mg/mL phenol, 15 mM trisodium citrate, pH 7.4). The pH of the resulting solution was adjusted to 7.60. The solution was filtered through a 0.22 **micron,** low protein binding filter. Five milliliters (5 mL) of protamine solution (37.9 mg of protamine sulfate in 50 mn of water) was added to 5 mL of the filtered insulin plus zinc solution. The resulting solution was allowed to stand undisturbed for 48 hours at a controlled **temperature** of 25.degree. C.

Detailed Description Text - DETX (209):

Microscopic inspection (at 48 hours) revealed that crystallization had occurred and that the preparation had yielded uniform, single, rod-like crystals possessing approximate average lengths of 5 **microns**.

Detailed Description Text - DETX (213):

The mean particle diameter of the crystals, determined as described in Preparation 14, was approximately 6 **microns,** with an approximately normal distribution, encompassing a range of particle sizes from approximately 2 **microns** to approximately 12 **microns**. This result is similar to the particle size distribution of commercial NPH as reported in DeFelippis, M. R., et al. supra.

Detailed Description Text - DETX (217):

To each of the four 0.4 mL solutions, 0.15 mL of a 1000 ppm zinc(II) solution was added. To each of the four 0.55 mL solutions, 1.6 mL of a crystallization diluent (50 mM tris buffer, 10 mg/mL phenol, 100 mM trisodium citrate, with a pH of 7.6) were added. Each of the four solutions was adjusted to pH 7.6 with small quantities of 1 N NaOH and 0.1 N HCl. Each solution was filtered through a 0.22 **micron,** low protein binding filter. Two milliliters (2 mL) of each of the four protein solutions were combined with 2 mL of protamine solution (7.34 mg of protamine sulfate in 10 mL of water). In each case, a precipitate formed immediately. These four 4 mL suspensions were allowed to stand undisturbed at room **temperature** (approximately 22.degree. C.) for 16 hours.

Detailed Description Text - DETX (218):

Microscopic inspection (at 16 hours) revealed that each of the four preparations had yielded uniform, single, rod-like crystals with approximate average lengths of about 10 **microns**.

Detailed Description Text - DETX (224):

A measured mass of a derivatized protein, prepared as described herein, was dissolved in 0.6 mL of 0.1 N HCl. A measured mass of a protein was dissolved in 0.2 mL of 0.1 N HCl (zinc crystals of human insulin or LysB28, Pro29-human insulin analog). The two solutions were thoroughly mixed together by stirring for five to ten minutes. A volume (0.32 mL) of an aqueous solution containing 1000 ppm Zn(II) and a volume (3.2 mL) of a diluent solution (about 50 mM Tris reagent, about 10 mg/ mL phenol, about 16 mg/mL glycerol, and about 29.5 mg/mL trisodium citrate) were added to the mixture of the two proteins. The pH of the resulting solution was adjusted to about 7.6 (7.55-7.64) using 1 N HCl or 1 N NaOH. The pH-adjusted solution was filtered through a 0.22 **micron,** low-protein binding filter. To four milliliters of the filtrate was added four milliliters of a solution of protamine in water (about 37.3 mg protamine sulfate per 100 mL, range 37.18-37.48). Precipitate formed immediately upon adding the protamine solution. The preparation was allowed to stand undisturbed at 25.degree. C. Dissolution tests were carried out as previously described. Under the same conditions, insulin NPH dissolved in about 6 minutes.

Detailed Description Text - DETX (226):

A measured mass of a derivatized protein, prepared as described herein, was dissolved in 3.2 mL of diluent solution (about 50 mM Tris reagent, about 10 mg/ mL phenol, about 16 mg/mL glycerol, and about 29.5 mg/mL trisodium citrate). A measured mass of a protein was dissolved in 0.6 mL of 0.1 N HCl (zinc crystals of human insulin or LysB28, Pro29-human insulin analog). The two solutions were thoroughly mixed together by stirring for five to ten minutes. The pH of the resulting solution was adjusted to about 7.6 (7.55-7.64) using 1 N HCl or 1 N NaOH. The pH-adjusted solution was filtered through a 0.22 **micron,** low-protein binding filter. To a volume of the filtrate was added an equal volume of a solution of protamine in water (about 37.3 mg protamine sulfate per 100 mL, range 37.18-37.48). Precipitate formed immediately upon adding the protamine solution. The preparation was allowed to stand undisturbed at 25.degree. C. Dissolution tests were carried out as previously described. Under the same conditions, insulin NPH dissolved in about 6 minutes.

Detailed Description Text - DETX (228):

A measured mass of a derivatized protein, prepared as described herein, was dissolved in a measured volume of 0.1 N HCl. A measured mass of a protein was dissolved in a measured volume of 0.1 N HCl (zinc crystals of human insulin or LysB28, Pro29-human insulin analog). Measured volumes of each of the two solutions were thoroughly mixed together by stirring for five to ten minutes. Measured volumes of an aqueous solution containing 1000 ppm Zn(II) and of a diluent solution (about 50 mM Tris reagent, about 10 mg/ mL phenol, about 32 mg/mL glycerol, and about 30 mg/mL trisodium citrate dihydrate, pH 8.47) were added to the mixture of the two proteins. The pH of the resulting solution was adjusted to about 7.6 (7.58-7.63) using 1 N HCl or 1 N NaOH. The pH-adjusted solution was filtered through a 0.22 **micron,** low-protein binding filter. To two milliliters of the filtrate was added two milliliters of a solution of protamine in water (about 37.5 mg protamine sulfate per 100 mL). Precipitate formed immediately upon adding the protamine solution. The preparation was allowed to stand undisturbed at 25.degree. C. Dissolution tests were carried out as previously described. Under the same conditions, insulin NPH dissolved in about 6 minutes.

Detailed Description Text - DETX (231):

A measured mass (13.84 mg of protein) of solid B28-tetradecanoyl-Lys(B28), Pro(B29) human insulin analog was dissolved in 0.375 mL of 0.1 N HCl. A measured mass of zinc human insulin (7.40 mg protein) was dissolved in 207 microliters of 0.1 N HCl. An aliquot (125 .mu.L) of the insulin solution (containing 4.47 mg of human insulin) was added to the solution of B28-tetradecanoyl-Lys(B28), Pro(B29)-human insulin analog. A volume (180 .mu.L) of 1000 ppm zinc and 2.0 mL of diluent (1.6 mg/mL phenol, 4 mg/mL m-cresol, 40 mg/mL glycerol, 5 mg/mL anhydrous sodium dibasic phosphate, 7.5 mg/mL trisodium phosphate dihydrate, pH 7.6) were added. The pH was increased from 5.6 to 8.0 with 100 microliters of 1N NaOH and back to 7.59 with 20 microliters of 1N HCl and 1N NaOH. The concentration of B28-tetradecanoyl-Lys(B28), Pro(B29) human insulin analog was 4.94 mg/mL and the human insulin concentration was 1.60 mg/mL. The solution was passed through a 0.22 **micron,** low-protein binding filter and refrigerated overnight. The next morning, the solution had no precipitate present. To 2.50 mL of the solution was added 2.88 mL of a protamine solution (0.75 mg/mL of solid protamine sulfate dissolved in water). An amorphous precipitate formed when the protamine was added.

Detailed Description Text - DETX (237):

A measured mass of solid derivatized protein was dissolved in 3 mL of 0.1 N HCl to produce a solution containing approximately 16 mg/mL derivatized protein. A measured mass of zinc human insulin crystals (73 mg, of which 67.17 mg was protein) was dissolved in 4.198 mL of 0.1 N HCl to produce a solution containing approximately 16 mg/mL insulin). Three milliliters of the solution of derivatized protein and one milliliter

of the insulin solution were combined and thoroughly mixed. Measured volumes of a 1000 ppm zinc solution (1.137 mL) and of a diluent (16 mL, containing, per mL: 1.625 mg phenol, 4 mg m-cresol, 40 mg glycerol, 5 mg anhydrous sodium dibasic phosphate, 7.5 mg trisodium citrate dihydrate, pH 7.6) were added. The pH was adjusted to about 7.6 (7.58-7.61) using 5 N NaOH and 5 N HCl solutions. The volume added during pH adjustment was from 0.11 to 0.12 mL. The solution was passed through a 0.22 **micron,** low-protein binding filter and refrigerated overnight. The next morning, the solution had no precipitate present. The solution was comprised of protein and derivatized protein (approximately a 1:3 mass ratio), and the total protein concentration was equivalent to about 85 units per milliliter. Just prior to testing in rats, equal volumes of the solution and of a solution of protamine sulfate (0.352 mg/mL) were combined and mixed thoroughly. An amorphous precipitate formed immediately. A sample of the suspension formulation containing the amorphous precipitate was promptly injected into test animals. After mixing with protamine, the concentration of total protein was about 42.4 units/ml.

Detailed Description Text - DETX (240):

Gly(A21)Arg(B31)Arg(B32)-human **insulin** was obtained from an E. coli **fermentation** in which a Gly(A21)-human proinsulin precursor molecule was overexpressed into inclusion bodies. A portion (94.7 g) of inclusion bodies was solubilized in 500 mL of 6 M guanidine hydrochloride containing 0.1 M TRIS, 0.27 M sodium sulfite, and 0.1 M sodium tetrathionate, pH 10.5 at room **temperature**. The pH was quickly lowered to 8.8 with 12 N HCl. After vigorously stirring in an open container for 45 minutes the pH was lowered to 2.1 with phosphoric acid and the sample centrifuged overnight at 4.degree. C. The supernatant was decanted and stored at 4.degree. C. for additional processing. The pellet was re-extracted with 200 mL of additional pH 10.5 solution (see above) and then centrifuged for 3 hours at 4.degree. C. This and the previously obtained supernatant were each diluted 4.times.with 100 mM sodium phosphate, pH 4, precipitating the product and other acidic components. After allowing the precipitate to settle, most of the supernatant was decanted and discarded. The resulting suspension was centrifuged, followed by decanting and discarding of additional supernatant, leaving wet pellets of the crude Gly(A21)-human proinsulin S-sulfonate precursor. The pellets were solubilized in 1.5 liters of 7 M deionized urea, adjusting the pH to 8 with 5 N NaOH and stirring over several hours at 4.degree. C. Salt (NaCl) was then added to achieve 1 M concentration and the sample was loaded onto a XAD-7 column (14 cm.times.20 cm, Toso-Haas, Montgomeryville, Pa.), previously flushed with 50% acetonitrile/50% 50 mM ammonium bicarbonate, 10% acetonitrile/90% 50 mM ammonium bicarbonate, and finally with 7 M deionized urea/1M NaCl/20 mM TRIS, pH 8. Once loaded, the column was pumped with 4.5 liters of a 7 M deionized urea/1 M NaCl/20 mM TRIS, pH 8 solution, followed by 2.8 liters of 50 mM ammonium bicarbonate/1 M NaCl, and 6.5 liters of 50 mM ammonium

bicarbonate. The column was eluted with a linear gradient of acetonitrile in 50 mM ammonium bicarbonate, while monitoring the eluant by UV at 280 nm. The peak of interest, partially purified Gly(A21)-human proinsulin S-sulfonate precursor, was collected, lyophilized, and subjected to a folding/disulfide bond procedure was dissolved in 3 liters of 20 mM glycine, pH 10.5, 4.degree. C. Then, 15 mL of 240 mM cysteine HCl were added with stirring, while maintaining the pH at 10.5 and the **temperature** at 4.degree. C. The reaction solution was stirred gently at 4.degree. C. for 27 hours and then quenched by lowering the pH to 3.1 with phosphoric acid. Acetonitrile (155 mL) was added, and the solution was then loaded onto a 5.times.25 cm C4 reversed-phase column previously pumped with 60% acetonitrile/40% water/0.1% TFA and equilibrated in 10% acetonitrile/90% water/0.1% TFA. Once loaded the column was pumped with 1 liter of 17.5% acetonitrile/82.5% water/0.1% TFA, then eluted with a linear gradient of acetonitrile in 0.1% TFA while monitoring at 280 nm. Selected fractions were pooled and lyophilized with a recovery of 714 mg. For conversion of the proinsulin precursor to the desired **insulin** analog, 697 mg of the Gly(A21) human proinsulin precursor were dissolved in 70 mL 50 mM ammonium bicarbonate, then chilled to 4.degree. C., pH 8.3. A volume (0.14 mL) of a 1 mg/mL solution of pork trypsin (Sigma Chemical Company, St. Louis, Mo.) in 0.01 N HCl was added to the sample solution which was stirred gently at 4.degree. C. for about 24 hours. An additional 0.14 mL of the trypsin solution was added to the reaction solution which was then stirred for an additional 21 hours, 45 minutes. The reaction was quenched by lowering the pH to 3.2 with 0.7 mL glacial acetic acid and 0.3 mL phosphoric acid. The quenched Gly(A21)Arg(B31)Arg(B32)-human **insulin** sample solution from the tryptic cleavage reaction was diluted 4.times.with 30% acetonitrile/70% 50 mM acetic acid, pH 3.1, and loaded onto a 1.times.30 cm S HyperD F (Biosepra, Marlborough, Mass.) cation exchange column previously pumped with 30% acetonitrile/70% 50 mM acetic acid/500 mM NaCl, pH 3.3, and equilibrated in 30% acetonitrile/70% 50 mM acetic acid. Once loaded the column was pumped with about 50 mL of 30% acetonitrile/70% 50 mM acetic acid, then eluted with a linear gradient of NaCl in 30% acetonitrile/50 mM acetic acid while monitoring the eluant at 276 nm. Selected fractions containing the Gly(A21)Arg(B31)Arg(B32)-human **insulin** were pooled, diluted 3.times.with purified water and loaded onto a 2.2.times.25 cm C4 reversed-phase column (Vydac, Hesperia, Calif.) previously pumped with 60% acetonitrile/40% water/0.1% TFA, then 10% acetonitrile/90% water/0.1% TFA. Once loaded, the column was pumped with about 200 mL of 10% acetonitrile/90% water/0.1% TFA, then eluted with a linear gradient of acetonitrile in 0.1% TFA. Selected fractions were pooled and lyophilized giving a recovery of 101 mg. Analytical HPLC revealed a purity of greater that 95% main peak. Electrospray mass spectroscopy (ESMS) analysis of the purified protein yielded a molecular weight of 6062.9 (6063.0, theory).

Detailed Description Text - DETX (243):

Des(B30)-human insulin was prepared from human proinsulin by controlled tryptic hydrolysis. A mass (2 g) of human proinsulin biosynthesized in recombinant E. coli and purified by conventional methods [Frank, B. H., et al., in PEPTIDES: Synthesis-Structure-Function. Proceedings of the Seventh American Peptide Symposium, Rich, D. H. and Gross, E. (Eds.), Pierce Chemical Company, Rockford, pp. 729-738, 1981; also, Frank, B. H., U.S. Pat. No. 4,430,266, issued Feb. 7, 1984, each of which is incorporated by reference] were dissolved in 400 mL of 0.1 M, pH 7.5 HEPES buffer. After addition of 8 mL of 1 M CaCl.sub.2 (in water) and pH adjustment to 7.5 with 5 N NaOH, 2 mL of a 10 mg/mL solution of pork trypsin (Sigma) in 0.01 N HCl were transferred to the sample solution while gently stirring. The reaction solution was allowed to stir at ambient **temperature** for 2 hours and 42 minutes, at which time it was transferred to a 37.degree. C. environment while stirring occasionally. After 1 hour and 45 minutes at 37.degree. C. the enzymatic reaction was quenched by lowering the pH to 3.0 with phosphoric acid and the **temperature** to 4.degree. C. for storage. Subsequently, the solution was brought to room **temperature** and diluted with 50 mL acetonitrile, then to a final volume of 500 mL with purified water, then loaded onto a 2.5.times.58 cm CG-161 (Toso-Haas) column previously pumped with 1 c.v. (column volume) of 40% acetonitrile/60% 0.1 M ammonium sulfate, pH 2.5, and 2 c.v. of 10% acetonitrile/90% 0.1 M ammonium sulfate, pH 2.5. Once loaded, the column was pumped with 1 c.v. of 10% acetonitrile/90% 0.1 M ammonium sulfate, pH 2.5. The column was eluted with a linear gradient of acetonitrile in 0.1 M ammonium sulfate, pH 2.5, while monitoring the eluant at 276 nm. The peak of interest, partially purified des(B30)-human insulin, was collected by pooling selected fractions. This pooled sample of partially purified des(B30)-human insulin was diluted to 1.28 liters with purified water, pH 3.5, and applied to a 1.times.29 cm S HyperD F (Biosepra) cation exchange column previously pumped with 1 c.v. of 30% acetonitrile/70% 0.1% TFA/0.5 M NaCl, pH 1.9, and 2 c.v. of 30% acetonitrile/70% 0.1% TFA, pH 2.3. Once loaded the column was pumped with 1 c.v. 30% acetonitrile/70% 0.1% TFA, pH 2.3, then eluted with a linear gradient of NaCl in 30% acetonitrile/70% 0.1% TFA, pH 1.9 to 2.3, while monitoring the eluant at 276 nm. Selected fractions containing the purified des(B30)-human insulin were pooled, diluted 2.5.times.with purified water and loaded onto a 35-c.c. C8 SepPak (Waters, Milford, Mass.) previously cleaned and primed with 2 c.v. of acetonitrile, 2 c.v. of 60% acetonitrile/40% 0.1% TFA, and 2 c.v. of 10% acetonitrile/90% 0.1% TFA. Once loaded the SepPak was flushed with 3 c.v. of 10% acetonitrile/90% 0.1% TFA and then eluted with 2 c.v. of 60% acetonitrile/40% 0.1% TFA. The lyophilized eluant yielded 500 mg. An analytical HPLC assay suggested greater than 95% main peak. Electrospray mass spectroscopy (ESMS) analysis of the purified protein yielded a molecular weight of 5706.5 (5707, theory).

Detailed Description Text - DETX (249):

Asp(B28)-human insulin was prepared and purified essentially according to the teaching of examples 31 and 32 of Chance, R. E., et al. (U.S. Pat. No. 5,700,662, issued Dec. 23,1997) which is expressly incorporated herein by reference. Des(B23-30)-human insulin [Bromer, W. W. and Chance, R. E., Biochim. Biophys. Acta, 133:219-223 (1967), which is incorporated herein by reference] and a synthetic octapeptide Gly-Phe-Phe-Tyr-Thr-Asp-Lys(Tfa)-Thr were condensed using trypsin-assisted semisynthesis, purified by gel **filtration** and reversed-phased HPLC, treated with 15% ammonium hydroxide (v/v) for four hours at ambient **temperature** to remove the trifluoroacetate (Tfa) blocking group from Lys(B29), purified by reversed-phase HPLC, and lyophilized.

Detailed Description Text - DETX (253):

A measured mass of purified insulin or of an insulin analog was dissolved in a measured volume of dimethylsulfoxide (DMSO) with stirring. Then, a measured volume of tetramethylguanidine hydrochloride (TMG) was added and the solution mixed thoroughly. In a separate container, a measured mass of an N-acyl-succinimide (NAS) was dissolved in a measured volume of DMSO. A measured volume of the second solution was added to the first solution. The reaction was carried out at room **temperature,** and the progress of the reaction was monitored by analyzing samples of the reaction mixture using HPLC. The reaction was quenched by adding a measured volume of ethanolamine, and then acidifying to pH 2-3.

Detailed Description Text - DETX (254):

The reaction mixture was then subjected to purification using reversed-phase chromatography alone, or using a combination of cation exchange chromatography followed by reversed-phase chromatography. The reversed-phase purification was carried out using an FPLC.RTM. system (Pharmacia) with UV detection at 214 nm or at 280 nm, a fraction collector, 2.2.times.25 cm or 5.times.30 cm C18 column, 2.5 or 5 mL/min flow rate, at room **temperature**. The liquid phases were mixtures of Solution A [0.1% trifluroacetic acid (TFA) in 10:90 acetonitrile:water (vol:vol)] and Solution B [0.1% trifluroacetic acid (TFA) in 70:30 acetonitrile:water (vol:vol)] appropriate to elute and separate the species of interest. Typically, the column was equilibrated and loaded while in 100% Solution A. Then, a linear gradient to some proportion of Solution B was used to separate the reaction products adequately. Fractions containing product were pooled. The development of purification methods is within the skill of the art.

Detailed Description Text - DETX (258):

A measured mass of purified insulin or of an insulin analog was dissolved by adding to it a measured volume of 50 mM boric acid, pH 2.57. A measured volume of acetonitrile, equal to the volume of boric acid solution, was then added slowly with stirring. The 'solvent" volume is the sum of the volumes of the boric acid and acetonitrile. The pH of

the solution was adjusted to between 10.2 and 10.5 using NaOH. In a separate container, a measured mass of an N-acyl-succinimide ("NAS") was dissolved in a measured volume of DMSO. A measured volume of the second solution was added to the first solution. The reaction was carried out at room **temperature,** the pH was maintained above 10.2 as necessary, and the progress of the reaction was monitored by analyzing samples of the reaction mixture using HPLC. The reaction was quenched by acidifying to pH 2-3. The reaction mixture was then subjected to purification using a reversed-phase chromatography system as described above.

Detailed Description Text - DETX (260):

The following is a general outline of a synthetic scheme to produce additional derivatized proteins. In a specific instance, the outline is to be read together with the data in Table 12, below, to a provide full synthetic scheme for a particular derivatized protein. A measured mass of purified insulin or insulin analog was dissolved by adding to it a measured volume DMSO. The pH of the solution was adjusted with 10 equivalents of tetramethylguanidine. In a separate container, a measured mass of an N-acyl-succinimide ("NASO") was dissolved in a measured volume of DMSO. A measured volume of the second solution was added to the first solution to provide a 1.9 fold molar excess of N-acyl-succinimide. The reaction was carried out at room **temperature** and the progress of the reaction was monitored by analyzing samples of the reaction mixture using HPLC. The reaction was quenched with 20 microliters of ethanolamine, chilled in ice/water bath and diluted 2.1 times with 0.1N HCl. The reaction mixture was then subjected to desalting on reversed phase chromatography column using the following protocol: 1) the column was wetted with 100% acetonitrile, then was washed using three to four column volumes of 0.1% TFA/70% acetonitrile (Buffer B); and finally was washed using four to five column volumes of 0.1% TFA/10% acetonitrile (Buffer A); 2) diluted reaction mixtures were loaded, and the column was again washed with five to six column volumes of Buffer A; and 3) the derivatized protein was eluted by passing five to six column volumes of Buffer B through the column. The fluid collected during elution was frozen, then lyophilized. The lyophilized crude product (86.1 mg) was then subjected to re-purification using a reversed-phase chromatography system as described above.

US-PAT-NO: 7125706

DOCUMENT-IDENTIFIER: US 7125706 B2

TITLE: Method for the production and purification of adenoviral vectors

DATE-ISSUED: October 24, 2006

PRIOR-PUBLICATION-INFORMATION:

DOCUMENT-IDENTIFIER	DOCUMENT-DATE
US 20020182723 A1	December 5, 2002

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APPL-NO: 09/880609

DATE FILED: June 12, 2001

CONTINUITY DATA:

division parent-doc US 09203078 00 19981201 PENDING child-doc US 09880609

continuation-in-part parent-doc US 08975519 00 19971129 PENDING child-doc US 09203078

us-provisional-application US 60031329 00 19961120

INT-CL-ISSUED:

TYPE	**IPC DATE IPC-OLD**
IPCP	C12N7/01 20060101 C12N007/01
IPCS	C12N15/861 20060101 C12N015/861
IPCS	A61K39/235 20060101 A61K039/235

INT-CL-CURRENT:

TYPE	**IPC DATE**
CIPP	C12 N 7/01 20060101
CIPS	A61 K 39/235 20060101
CIPS	C12 N 15/861 20060101

US-CL-ISSUED: 435/235.1 , 435/239 , 435/320.1 , 424/199.1 , 424/233.1 , 424/93.2

US-CL-CURRENT: 435/235.1, 424/199.1 , 424/233.1 , 424/93.2 , 435/239 , 435/320.1

FIELD-OF-CLASSIFICATION-SEARCH: 435/235.1; 435/239 ; 435/320.1 ; 424/199.1 ; 424/233.1 ; 424/93.2

****See application file for complete search history****

REF-CITED:

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ART-UNIT: 1648

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ABSTRACT:

The present invention addresses the need to improve the yields of viral vectors when grown in cell culture systems. In particular, it has been demonstrated that for adenovirus, the use of low-medium perfusion rates in an attached cell culture system provides for improved yields. In other embodiments, the inventors have shown that there is improved Ad-p53 production cells grown in serum-free conditions, and in particular in serum-free suspension culture. Also important to the increase of yields is the use of detergent lysis. Combination of these aspects of the invention permits purification of virus by a single chromatography step that results in purified virus of the same quality as preparations from double CsCl banding using an ultracentrifuge.

20 Claims, 72 Drawing figures

Exemplary Claim Number: 1 2

Number of Drawing Sheets: 49

Description Paragraph - DETX (77):

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. Large scale suspension culture based on microbial (bacterial and yeast) **fermentation** technology has clear advantages for the manufacturing of mammalian cell products. The processes are relatively simple to operate and straightforward to scale up. Homogeneous conditions can be provided in the reactor which allows for precise monitoring and control of temperature, dissolved oxygen, and pH, and ensures that representative samples of the culture can be taken.

Description Paragraph - DETX (80):

Large scale suspension culture of mammalian cells in stirred tanks was undertaken. The instrumentation and controls for bioreactors adapted, along with the design of the fermentors, from related microbial applications. However, acknowledging the increased demand for contamination control in the slower growing mammalian cultures, improved aseptic designs were quickly implemented, improving dependability of these reactors. Instrumentation and controls are basically the same as found in other fermentors and include agitation, temperature, dissolved oxygen, and pH controls. More advanced probes and autoanalyzers for on-line and off-line measurements of turbidity (a function of particles present), capacitance (a function of viable cells present), glucose/lactate, carbonate/bicarbonate and carbon dioxide are available. Maximum cell densities obtainable in suspension cultures are relatively low at about 2 4.times.10.sup.6 cells/ml of medium (which is less than 1 mg dry cell weight per ml), well below the numbers achieved in microbial **fermentation**.

Description Paragraph - DETX (82):

The airlift reactor, also initially described for microbial **fermentation** and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easily, has good mass transfer of gasses and generates relatively low shear forces.

Description Paragraph - DETX (85):

In what is still a closed system, perfusion of fresh medium through the culture can be achieved by retaining the cells with a variety of devices (e.g. fine mesh spin filter, hollow fiber or flat plate **membrane** filters, settling tubes). Spin filter cultures can produce cell densities of approximately 5.times.10.sup.7 cells/ml. A true open system and the simplest perfusion process is the chemostat in which there is an inflow of medium and an outflow of cells and products. Culture medium is fed to the reactor at a predetermined and constant rate which maintains the dilution rate of the culture at a value less than the maximum specific growth rate of the cells (to prevent washout of the cell mass from the reactor). Culture fluid containing cells and cell products and byproducts is removed at the same rate.

Description Paragraph - DETX (87):

Traditionally, anchorage-dependent cell cultures are propagated on the bottom of small glass or plastic vessels. The restricted surface-to-volume ratio offered by classical and traditional techniques, suitable for the laboratory scale, has created a bottleneck in the production of cells and cell products on a large scale. In an attempt to provide systems that offer large accessible surfaces for cell growth in small culture volume, a number of techniques have been proposed: the roller bottle system, the stack plates propagator, the spiral film bottles, the hollow fiber system, the packed bed, the plate exchanger system, and the **membrane** tubing reel. Since these systems are non-homogeneous in their nature, and are sometimes based on multiple processes, they suffer from the following shortcomings--limited potential for scale-up, difficulties in taking cell samples, limited potential for measuring and controlling key process parameters and difficulty in maintaining homogeneous environmental conditions throughout the culture.

Description Paragraph - DETX (93):

One method which has shown to be particularly useful for culturing mammalian cells is microencapsulation. The mammalian cells are retained inside a semipermeable hydrogel **membrane**. A porous **membrane** is formed around the cells permitting the exchange of nutrients, gases, and metabolic products with the bulk medium surrounding the capsule. Several methods have been developed that are gentle, rapid and non-toxic and where the resulting **membrane** is sufficiently porous and strong to sustain the growing cell mass throughout the term of the culture. These methods are all based on soluble alginate gelled by droplet contact with a calcium-containing solution. Lim (1982, U.S. Pat. No. 4,352,883, incorporated herein by reference,) describes cells concentrated in an approximately 1% solution of sodium alginate which are forced through a small orifice, forming droplets, and breaking free into an approximately 1% calcium chloride solution. The droplets are then cast in a layer of polyamino acid that ionically bonds to the surface alginate. Finally the alginate is reliquefied by treating the droplet in a chelating agent to

remove the calcium ions. Other methods use cells in a calcium solution to be dropped into a alginate solution, thus creating a hollow alginate sphere. A similar approach involves cells in a chitosan solution dropped into alginate, also creating hollow spheres.

Description Paragraph - DETX (115):

Cells are bounded by **membranes**. In order to release components of the cell, it is necessary to break open the cells. The most advantageous way in which this can be accomplished, according to the present invention, is to solubilize the **membranes** with the use of detergents. Detergents are amphipathic molecules with an apolar end of aliphatic or aromatic nature and a polar end which may be charged or uncharged. Detergents are more hydrophilic than lipids and thus have greater water solubility than lipids. They allow for the dispersion of water insoluble compounds into aqueous media and are used to isolate and purify proteins in a native form.

Description Paragraph - DETX (116):

Detergents can be denaturing or non-denaturing. The former can be anionic such as sodium dodecyl sulfate or cationic such as ethyl trimethyl ammonium bromide. These detergents totally disrupt **membranes** and denature the protein by breaking protein-protein interactions. Non denaturing detergents can be divided into non-anionic detergents such as Triton.RTM.X-100, bile salts such as cholates and zwitterionic detergents such as CHAPS. Zwitterionics contain both cationic and anion groups in the same molecule, the positive electric charge is neutralized by the negative charge on the same or adjacent molecule.

Description Paragraph - DETX (121):

Triton.RTM.X-Detergents: This family of detergents (Triton.RTM.X-100, X114 and NP-40) have the same basic characteristics but are different in their specific hydrophobic-hydrophilic nature. All of these heterogeneous detergents have a branched 8-carbon chain attached to an aromatic ring. This portion of the molecule contributes most of the hydrophobic nature of the detergent. Triton.RTM.X detergents are used to solublize **membrane** proteins under non-denaturing conditions. The choice of detergent to solubilize proteins will depend on the hydrophobic nature of the protein to be solubilized. Hydrophobic proteins require hydrophobic detergents to effectively solubilize them.

Description Paragraph - DETX (122):

Triton.RTM. X-100 and NP40 are very similar in structure and hydrophobicity and are interchangeable in most applications including cell lysis, delipidation protein dissociation and **membrane** protein and lipid solubilization. Generally 2 mg detergent is used to

solubilize lmg **membrane** protein or 10 mg detergent/lmg of lipid **membrane**. Triton.RTM. X-114 is useful for separating hydrophobic from hydrophilic proteins.

Description Paragraph - DETX (124):

Dializable Nonionic Detergents: .eta.-Octyl-.beta.-D-glucoside (octylglucopyranoside) and .eta.-Octyl-.beta.-D-thioglucoside (octylthioglucopyranoside, OTG) are nondenaturing nonionic detergents which are easily dialyzed from solution. These detergents are useful for solubilizing **membrane** proteins and have low UV absorbances at 280 nm. Octylglucoside has a high CMC of 23 25 mM and has been used at concentrations of 1.1 1.2% to solubilize **membrane** proteins.

Description Paragraph - DETX (127):

Tween.RTM. 20 and other nonionic detergents have been shown to remove some proteins from the surface of nitrocellulose. Tween.RTM. 80 has been used to solubilize **membrane** proteins, present nonspecific binding of protein to multiwell plastic tissue culture plates and to reduce nonspecific binding by serum proteins and biotinylated protein A to polystyrene plates in ELISA.

Description Paragraph - DETX (130):

Zwitterionic Detergents: The zwitterionic detergent, CHAPS, is a sulfobetaine derivative of cholic acid. This zwitterionic detergent is useful for **membrane** protein solubilization when protein activity is important. This detergent is useful over a wide range of pH (pH 2 12) and is easily removed from solution by dialysis due to high CMCs (8 10 mM). This detergent has low absorbances at 280 nm making it useful when protein monitoring at this wavelength is necessary. CHAPS is compatible with the BCA Protein Assay and can be removed from solution by detergent removing gel. Proteins can be iodinated in the presence of CHAPS.

Description Paragraph - DETX (131):

CHAPS has been successfully used to solubilize intrinsic **membrane** proteins and receptors and maintain the functional capability of the protein. When cytochrome P-450 is solubilized in either Triton.RTM. X-100 or sodium cholate aggregates are formed.

Description Paragraph - DETX (143):

One aspect of the present invention employs methods of crude purification of adenovirus from a cell lysate. These methods include clarification, concentration and diafiltration. The initial step in this purification process is clarification of the cell lysate to remove large particulate matter, particularly cellular components, from the cell lysate.

Clarification of the lysate can be achieved using a depth filter or by tangential flow filtration. In a preferred embodiment of the present invention, the cell lysate is passed through a depth filter, which consists of a packed column of relatively non-adsorbent material (e.g. polyester resins, sand, diatomeceous earth, colloids, gels, and the like). In tangential flow filtration (TFF), the lysate solution flows across a **Membrane** surface which facilitates back diffusion of solute from the **membrane** surface into the bulk solution. **Membranes** are generally arranged within various types of filter apparatus including open channel plate and frame, hollow fibers, and tubules.

Description Paragraph - DETX (144):

After clarification and prefiltration of the cell lysate, the resultant virus supernatant is first concentrated and then the buffer is exchanged by diafiltration. The virus supernatant is concentrated by tangential flow filtration across an ultrafiltration **membrane** of 100 300K nominal molecular weight cutoff. Ultrafiltration is a pressure-modified convective process that uses semi-permeable **membranes** to separate species by molecular size, shape and/or charge. It separates solvents from solutes of various sizes, independent of solute molecular size. Ultrafiltration is gentle, efficient and can be used to simultaneously concentrate and desalt solutions. Ultrafiltration **membranes** generally have two distinct layers: a thin (0.1 1.5 .mu.m), dense skin with a pore diameter of 10 400 angstroms and an open substructure of progressively larger voids which are largely open to the permeate side of the ultrafilter. Any species capable of passing through the pores of the skin can therefore freely pass through the **membrane**. For maximum retention of solute, a **membrane** is selected that has a nominal molecular weight cut-off well below that of the species being retained. In macromolecular concentration, the **membrane** enriches the content of the desired biological species and provides filtrate cleared of retained substances. Microsolutes are removed convectively with the solvent. As concentration of the retained solute increases, the ultrafiltration rate diminishes.

Description Paragraph - DETX (200):

C-CAM is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kD, was originally isolated from the plasma **membrane** of the rat hepatocyte by its reaction with specific antibodies that neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung et al. (1993a; 1993b and 1993c) demonstrated that the first Ig domain of C-CAM is critical for cell adhesion activity.

Description Paragraph - DETX (204):

Hormones are another group of gene that may be used in the vectors described herein. Included are growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropin (ACTH), angiotensin I and II, .beta.-endorphin, .beta.-melanocyte stimulating hormone (.beta.-MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, **insulin,** lipotropins, neurophysins, somatostatin, calcitonin, calcitonin gene related peptide (CGRP), .beta.-calcitonin gene related peptide, hypercalcemia of malignancy factor (1 40), parathyroid hormone-related protein (107 139) (PTH-rP), parathyroid hormone-related protein (107 111) (PTH-rP), glucagon-like peptide (GLP-1), pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalinamide, metorphinamide, alpha melanocyte stimulating hormone (alpha-MSH), atrial natriuretic factor (5 28) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P and thyrotropin releasing hormone (TRH).

Description Paragraph - DETX (222):

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, .beta.-actin, rat **insulin** promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

Description Paragraph - DETX (228):

TABLE-US-00003 TABLE 3 Tissue specific promoters Tissue Promoter Pancreas **Insulin** elastin amylase pdr-1 pdx-1 glucokinase Liver albumin PEPCK HBV enhancer alpha fetoprotein apolipoprotein C alpha-1 antitrypsin vitellogenin, NF-AB Transthyretin Skeletal muscle myosin H chain muscle creatine kinase dystrophin calpain p94 skeletal alpha-actin fast troponin 1 Skin keratin K6 keratin K1 Lung CFTR human cytokeratin 18 (K18) pulmonary surfactant proteins A, B and C CC-10 P1 Smooth muscle sm22 alpha SM-alpha-actin Endothelium endothelin-1 E-selectin von Willebrand factor TIE (Korhonen et al., 1995) KDR/flk-1 Melanocytes Tyrosinase Adipose tissue lipoprotein lipase (Zechner et al., 1988) adipsin (Spiegelman et al., 1989) acetyl-CoA carboxylase (Pape and Kim, 1989) glycerophosphate dehydrogenase (Dani et al., 1989) adipocyte P2 (Hunt et al., 1986) Blood .beta.-globin

Description Paragraph - DETX (233):

TABLE-US-00004 TABLE 4 PROMOTER Immunoglobulin Heavy Chain Immunoglobulin Light Chain T-Cell Receptor HLA DQ .alpha. and DQ .beta. .beta.-Interferon Interleukin-2 Interleukin-2 Receptor MHC Class II 5 MHC Class II HLA-DR.alpha. .beta.-Actin Muscle Creatine Kinase Prealbumin (Transthyretin) Elastase I Metallothionein Collagenase Albumin Gene .alpha.-Fetoprotein .tau.-Globin .beta.-Globin c-fos c-HA-ras **Insulin** Neural Cell Adhesion Molecule (NCAM) .alpha.1-Antitrypsin H2B (TH2B) Histone Mouse or Type I Collagen Glucose-Regulated Proteins (GRP94 and GRP78) Rat Growth Hormone Human Serum Amyloid A (SAA) Troponin I (TN I) Platelet-Derived Growth Factor Duchenne Muscular Dystrophy SV40 Polyoma Retroviruses Papilloma Virus Hepatitis B Virus Human Immunodeficiency Virus Cytomegalovirus Gibbon Ape Leukemia Virus

Description Paragraph - DETX (246):

In one embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell **membrane**. This is particularity applicable for transfer in vitro, however, it may be applied for in vivo use as well. Dubensky et al. (1984) successfully injected polyomavirus DNA in the form of CaPO.sub.4 precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of CaPO.sub.4 precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner in vivo and express CAM.

Description Paragraph - DETX (247):

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell **membranes** and enter cells without killing them (Klein et al., 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang et al., 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Description Paragraph - DETX (248):

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer

membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991).

Description Paragraph - DETX (250):

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell **membrane** and promote cell entry of liposome-encapsulated DNA (Kaneda et al., 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato et al., 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid in vitro and in vivo, then they are applicable for the present invention.

Description Paragraph - DETX (253):

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau et al. (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the **insulin** gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a therapeutic gene also may be specifically delivered into a cell type such as prostate, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt et al., 1986) may be used as the receptor for mediated delivery of a nucleic acid in prostate tissue.

Description Paragraph - DETX (313):

Sterility assays (documented in U.S.P. XXIII <71>) are used at both the bulk and final product stage. Sterility testing is via **membrane** filtration and is performed in a soft-wall isolator system to minimize laboratory contamination of samples tested. All test articles should preferably pass the sterility test.

Description Paragraph - DETX (315):

The bioburden test is used to detect microbial load in a test sample by filtering the test sample onto a **membrane** filter, placing the **membrane** filter onto Tryptic Soy agar and Sabourad agar plates and observing for growth after 2 5 days incubation. Suspensions with known levels of Bacillus subtilis and Candid albicans are also assayed to confirm assay suitability.

Description Paragraph - DETX (316):

Briefly the test method is as follows. Test samples may be stored up to 24 hours at 2 8 degree Celsius before testing. Reserve samples that are not to be tested within 24 hours may be frozen at less than -60 degrees Celsius. Negative controls (sterile diluent) are prepared by filtering 100 mL of sterile diluent through an analytical filter unit using a vacuum. The **membrane** filter is removed from the unit and placed on a pre-warmed Tryptic Soy agar plate. The process is repeated using a second filter unit and the filter is placed on a pre-warmed Sabouraud agar plate. In-process test samples are tested by filtering 5.times.10 mL of crude cell lysate onto 5 separate filters or 10 mL of prefiltered bulk product onto a single filter. Each **membrane** filter is removed from the unit and placed on a pre-warmed Tryptic Soy agar plate. The process is repeated using a second set of filter units and the filter is placed on a pre-warmed Sabouraud agar plate. Bacillus subtilis positive controls are prepared by filtering 50 mL of sterile diluent through an analytical filter unit using a vacuum. The **membrane** filter is removed from the unit and placed on a pre-warmed Tryptic Soy agar plate. The process is repeated using a Candida albicans positive control using a second filter unit and the filter is placed on a pre-warmed Sabouraud agar plate. Tryptic Soy agar plates are incubated at 30 35 degrees Celsius for 2 5 days. Sabouraud agar plates are incubated at 22 27 degrees Celsius for 5 7 days. Colonies on the **membrane** filters are counted after the incubation period. The assays are acceptable when the negative controls exhibit no growth and positive controls exhibit 1 100 colonies per **membrane** filter. The test article should preferably contain less than or equal to 1000 colony forming units per 100 mL of the crude cell lysate. It is more preferable that the crude cell lysate contain less than or equal to 500 colony forming units per 100 mL, and most preferable that the crude cell lysate contain less than or equal to 10 colony forming units per 100 mL. It is most preferable that the prefiltered bulk product contain less than or equal to one colony forming unit per 10 mL. Using purification techniques in accordance with the present disclosure, bioburden values less than 1 have been obtained at the crude cell lysate step, and less than 1 at the prefiltered bulk product step.

Description Paragraph - DETX (322):

This assay detects the presence of Mycoplasma in a test article based on the ability of Mycoplasma to grow in any one of the test systems: Agar isolation and Vero cell culture system. Growth is signified by colony formation, shift in pH indicators, or presence of Mycoplasma by staining, depending on the system used. The assay is performed using a large sample volume. The test methods are as follows. The test article and positive controls are inoculated directly onto Mycoplasma agar plates and into Mycoplasma semi-solid **broth** which is subcultured three times onto agar plates. The samples are incubated both aerobically and anaerobically. At 14 days post-infection the agar plates are examined for evidence of growth. The test article is also inoculated directly onto Vero

cell cultures and incubated for 3 5 days. The cultures are stained with a DNA-binding fluorochrome and evaluated microscopically by epifluorescence for the presence of Mycoplasma.

Description Paragraph - DETX (323):

For the Agar isolation assay, the positive controls should preferably show Mycoplasma growth in at least two out of five direct plates for each media type and for each incubation condition, and in the semi **-broth**. The negative control plates and bottles should preferably show absence of Mycoplasma growth. For the Vero cell culture assay, positive controls should preferably show the presence of Mycoplasma, negative controls should preferably show no presence of Mycoplasma, and all of the controls should preferably show the absence of bacterial or fungal contaminants. The test article will preferably be negative for the presence of Mycoplasma.

Description Paragraph - DETX (354):

The test method is as follows. Total proteins are determined using a Pierce BCA method according to the protocol described previously in this section. The test sample, internal standard and molecular weight standards are prepared in sample buffer and denatured by **heating**. All samples and standards are loaded into wells of a pre-cast Tris-glycine gel and set in an electrophoresis tank containing running buffer. The gel is run on a constant current setting for approximately 90 minutes. The gel is then removed from the cassette, stained using Coomassie Brilliant Blue stain and destained. The gel is then analyzed using a densitometric scanning instrument, and the data captured by photography. Alternatively, the gel is dried for archiving. In all controls, the presence of expected proteins is preferable and there should preferably be no contaminating proteins. In the test sample, the expected bands should preferably be observed, with no significant extra bands.

Description Paragraph - DETX (356):

This method tests for the presence of p53 protein in Ad5CMV-p53 transduced cells. The test method is as follows. Individual 60 mm tissue culture dishes for product samples and control samples are seeded at a density of 7.times.10.sup.5 cells and grown to greater than 80% confluence. The test article is diluted in media to provide 3.5.times.10.sup.8 viral particles/mL. A reference control is diluted to 3.5.times.10.sup.8 vp/mL and a negative control with no vector is also prepared. The cells are exposed to media containing product for one hour during which the plates are rocked to ensure even distribution of vector. At the end of the hour, additional media is added to the dishes and they are incubated for approximately five hours to allow time for expression of p53. At the end of the incubation period, the cells are treated with trypsin to allow harvest, washed with DPBS and solubilized with a detergent buffer. The total amount of protein

in each sample and control is determined by a colorimetric quantitation method (Pierce BCA). For each sample and method, 3 5 micrograms of protein are loaded onto a gel alongside a commercially purchased p53 protein reference and separated by polyacrylamide gel electrophoresis (PAGE). The proteins in the gel are transferred to a PVDF **membrane and the membrane** is exposed to a milk buffer to block non-specific binding sites and then sequentially exposed to antibodies. The primary antibody, a mouse anti-human p53 antibody specifically binds to p53. The secondary antibody is a goat anti-mouse IgG with horseradish peroxidase (HRP) covalently bound. A colorimetric substrate is exposed to the bound HRP enzyme which enables visualization of p53 protein on the blot. For the assay to be considered valid, the control p53 band should preferably be visible, and the negative control should preferably show no expression of p53. The test article should preferably show expression of p53.

Description Paragraph - DETX (430):

Virus solution from the lysis step was clarified and filtered before concentration/diafiltration. TFF **membranes** of different NMWCs, including 100K, 300K, 500K, and 1000K, were evaluated for efficient concentration/diafiltration. The highest medium flux with minimal virus loss to the filtrate was obtained with a **membrane** of 300K NMWC. Bigger NMWC **membranes** offered higher medium flux, but resulted in greater virus loss to the filtrate, while smaller NMWC **membranes** achieved an insufficient medium flux. Virus solution was first concentrated 10-fold, which was followed by 4 sample volumes of diafiltration against 20 mM Tris+0.25 M NaCl+1 mM MgCl.sub.2, pH=9.00 buffer using the constant volume method. During the concentration/diafiltration process, pressure drop across the **membrane** was kept .ltoreq.5 psi. Consistent, high level virus recovery was demonstrated during the concentration/diafiltration step as indicated in Table 10.

Description Paragraph - DETX (477):

A concentration/diafiltration step after column purification serves not only to increase the virus titer, if necessary, but also to exchange to the buffer system specified for the virus product. A 300K NMWC TFF **membrane** was employed for the concentration step. Because of the absence of proteinacious and nucleic acid contaminants in the purified virus, very high buffer flux was achieved without noticeable pressure drop across the **membrane**.

Description Paragraph - DETX (531):

In particular, in terms of industrialization, the process according to the invention uses methods of the treatment of supernatants of cultures tested on a large scale for recombinant proteins, such as **microfiltration** or deep filtration, and tangential ultrafiltration. Furthermore, because of the stability of the virus at 37.degree. C., this

process permits better organization at the industrial stage inasmuch as, contrary to the intracellular method, the harvesting time does not need to be precise to within a half day. Moreover, it guarantees maximum harvesting of the virus, which is particularly important in the case of viruses defective in several regions. In addition, the process according to the invention permits an easier and more precise follow-up of the production kinetics directly on homogenous samples of supernatant, without pretreatment, which permits better reproducibility of the productions. The process according to the invention also makes it possible to eliminate the cell lysis step. The lysis of the cells presents a number of drawbacks. Thus, it may be difficult to consider breaking the cells by freeze/thaw cycles at the industrial level. Besides, the alternative lysis methods (Dounce, X-press, sonification, mechanical shearing, etc.) present drawbacks as well: they are potential generators of sprays that are difficult to confine for L2 or L3 viruses (level of confinement of the viruses, depending on their pathogenicity of their mode of dissemination), with these viruses having a tendency to be infectious through airborne means; they generate shear forces and/or a liberation of heat that are difficult to control, diminishing the activity of the preparations. The solution of using detergents to lyse the cells would demand validation and would also require that elimination of the detergent be validated. Finally, cellular lysis leads to the presence in the medium of a large quantity of cellular debris, which makes purification more difficult. In terms of virus quality, the process according to the invention potentially permits better maturation of the virus, leading to a more homogenous population. In particular, provided that the packing of the viral DNA is the last step in the viral cycle, the premature lysis of the cells potentially liberates empty particles which, although not replicative, are a priori infectious and capable of participating in the distinctive toxic effect of the virus and of increasing the ratio of specific activity of the preparations obtained. The ratio of specific infectivity of a preparation is defined as the ratio of the total number of viral particles, measured by biochemical methods (OD 260 nm, HPLC, CRP, immuno-enzymatic methods, etc.), to the number of viral particles generating a biologic effect (formation of lysis plaques on cells in culture and solid medium, translation of cells). In practice, for a purified preparation, this ratio is determined by dividing the concentration of particles measured by OD at 260 nm by the concentration of plaque-forming units in the preparation. This ratio should be less than 100.

Description Paragraph - DETX (540):

To recover the viral particles, the culture supernatant is advantageously first filtered. Since the adenovirus is approximately 0.1 .mu.m (120 nm) in size, filtration is performed with **membranes** whose pores are sufficiently large to let the virus pass through, but sufficiently fine to retain the contaminants. Preferably, filtration is performed with **membranes** having a porosity greater than 0.2 .mu.m. According to a particularly advantageous exemplified embodiment, filtration is performed by successive filtrations on **membranes** of decreasing porosity. Particularly good results have been obtained by

doing filtration on filters with a range of decreasing porosity--10 .mu.m, 1.0 .mu.m, then 0.8 0.2 .mu.m. According to another preferred variant, filtration is performed by tangential **microfiltration** on flat **membranes** or hollow fibers. More particularly, it is possible to use flat Millipore **membranes** or hollow fibers ranging in porosity between 0.2 and 0.6 .mu.m. The results presented in the examples show that this filtration step has a yield of 100% (no loss of virus was observed by retention on the filter having the lowest porosity).

Description Paragraph - DETX (541):

According to another aspect of the invention, the applicant has now developed a process making it possible to harvest and purify the virus from the supernatant. Toward this goal, a supernatant thus filtered (or clarified) is subjected to ultrafiltration. This ultrafiltration makes is possible (i) to concentrate the supernatant, with the volumes used being important; (ii) to do a first purification of the virus and (iii) to adjust the buffer of the preparation in the subsequent preparation steps. According to a preferred exemplified embodiment, the supernatant is subjected to tangential ultrafiltration. Tangential ultrafiltration consists of concentrating and fractionating a solution between two compartments, retentate and filtrate, separated by **membranes** of specified cutoff thresholds, by producing a flow in the retentate compartment of the apparatus and by applying a transmembrane pressure between this compartment and the filtrate compartment. The flow is generally produced with a pump in the retentate compartment of the apparatus, and the transmembrane pressure is controlled by a valve on the liquid channel of the retentate circuit or by a variable-speed pump on the liquid channel of the filtrate circuit. The speed of the flow and the transmembrane pressure are chosen so as to generate low shear forces (Reynolds number less than 5000 sec.sup.-1, preferably below 3000 sec.sup.-1, pressure below 1.0 bar), while preventing plugging of the **membranes**. Different systems can be used to accomplish ultrafiltration, e.g., spiral **membranes** (Millipore, Amicon), as well as flat **membranes** or hollow fibers (Amicon, Millipore, Sartorius, Pall, GF, and Sepracor). Since the adenovirus has a mass of ca. 1000 kDa, it is advantageous within the scope of the invention to use **membranes** having a cutoff thresh below 1000 kDa, and preferably ranging between 100 kDa and 1000 kDa. The use of **membranes** having a cutoff threshold of 1000 kDa or higher in effect causes a large loss of virus at this stage. It is preferable to use **membranes** having a cutoff threshold ranging between 200 and 600 kDa, and even more preferable, between 300 and 500 kDa. The experiences presented in the examples show that the use of a **membrane** having a cutoff threshold at 300 kDa permits more than 90% of the viral particles to be retained, while eliminating the contaminants from the medium (DNA, proteins in the medium, cellular proteins, etc.). The use of a cutoff threshold of 500 kDa offers the same advantages.

Description Paragraph - DETX (543):

This ultrafiltration step thus includes an additional purification compared to the classical model inasmuch as the contaminants of mass below the cutoff threshold (300 or 500 kDa) are eliminated at least in part. A distinct improvement in the quality of the viral preparation may be seen upon comparing the appearance of the separation after the first ultracentrifugation step according to the two processes. In the classical process involving lysis, the viral preparation tube presents a cloudy appearance with a coagulum (lipids, proteins) sometimes touching the virus band, while in the process according to the invention, following liberation and ultrafiltration, the preparation presents a band that is already well resolved of the contaminants of the medium that persist in the upper phase. An improvement in quality is also demonstrated upon comparing the profiles on ion exchange chromatography of a virus obtained by cellular lysis with a virus obtained by ultrafiltration as described in the present invention. In addition, it is possible to further enhance the quality by pursuing ultrafiltration with diafiltration of the concentrate. This diafiltration is performed based on the same principle as tangential ultrafiltration, and makes it possible to more completely eliminate the large-sized contaminants at the cutoff threshold of the **membrane,** while achieving equilibration of the concentrate in the purification buffer.

Description Paragraph - DETX (578):

Previous studies looking at virus release kinetics after Ad5CMV-p53 infection of 293 cells determined that maximal virus release from the producer cells due to the lytic nature of adenovirus was obtained four to six days after infection. Thus, four to six days after virus infection, the supernatant from the Cellcube.TM. modules was removed as a pool. The virus supernatant was then clarified by filtration through two Polyguard 5.0 micron filters, followed by a 5.0 micron Polysep filter (Millipore). The supernatant was then concentrated approximately 10-fold using tangential flow filtration through a Pellicon cassette (Millipore) of 300 K nominal molecular weight cut-off (NMWC). The buffer was then exchanged by diafiltration against 0.5 M Tris+1 mM MgCl.sub.2, pH=8. The supernatant was then treated at room temperature with 100 U/ml Benzonase.TM. in a buffer of 0.5M Tris/HCl+1 mM MgCl.sub.2, pH=8.0; 0.2 micron filtered, and incubated overnight at room temperature to remove contaminating cellular nucleic acids. The crude virus preparation is then 0.2 micron filtered and loaded directly onto an ion exchange column (BPG 200/500, Pharmacia) containing Source 15Q resin equilibrated with 20 mM Tris+1 mM MgCl.sub.2+250 mM NaCl, pH=8.0. The virus was eluted with a 40 column linear gradient using an elution buffer composed of 20 mM Tris+1 mM MgCl.sub.2+2 M NaCl, pH=8.0. The purified virus was then subjected to another concentration and diafiltration step to place the virus in the final formulation for the virus product. The concentration step used a 300 NMWC Pellicon TFF **membrane,** and for diafiltration the buffer was exchanged using 8 10 column volumes of Dulbecco's Phosphate Buffered Saline+10% Glycerol. The purified virus was then sterile filtered through a 0.2 micron Millipak (Millipore) filter. The formulated product was then filled

into sterile glass vials with stoppers. Flip off crimp caps were applied prior to final product inspection and labeling.

Other Reference Publication - OREF (69):

Lin and Guidotti, "Cloning and expression of a cDNA coding for a rat liver plasma **membrane** ecto-ATpase," J. Biol. Chem., 264:14408-14414, 1989. cite- d by other

US-PAT-NO:	5202239
DOCUMENT-IDENTIFIER:	US 5202239 A
TITLE:	Expression of recombinant polypeptides with improved purification
DATE-ISSUED:	April 13, 1993

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APPL-NO: 07/564259
DATE FILED: August 7, 1990

INT-CL-ISSUED: [05] C12N015/62 , C12N015/12

INT-CL-CURRENT:

TYPE	IPC DATE
CIPS	C07 K 14/58 20060101
CIPS	C07 K 14/435 20060101
CIPS	C12 N 15/62 20060101

US-CL-ISSUED:	435/69.7 , 435/172.3 , 435/252.3 , 435/320.1 , 530/350 , 530/412
US-CL-CURRENT:	435/69.7, 435/252.3 , 435/320.1 , 530/350 , 530/412
FIELD-OF-CLASSIFICATION-SEARCH:	435/69.7; 435/172.3 ; 530/350 ; 530/412 ; 935/47

****See application file for complete search history****

REF-CITED:

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4532207	July 1985	Brewer et al.	435/69.4	N/A	N/A
4743679	May 1988	Cohen et al.	530/350	N/A	N/A
4764504	August 1988	Johnson et al.	514/12	N/A	N/A
4880911	November 1989	Brewer et al.	530/351	N/A	N/A
4897348	January 1990	Johnson et al.	435/69.1	N/A	N/A
4987070	January 1991	Magota et al.	435/69.7	N/A	N/A

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FOREIGN-PAT-NO	PUBN-DATE	COUNTRY	US-CL
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2180539	April 1987	GB	

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Sung et al., Proc. Natl. Acad. Sci. (1986) 83:561-565.

Cockle et al., "Protein Purification: Micro to Macro" (1987)

Alan R. Liss, Inc., pp. 375-381.

Sassenfeld et al., Bio/Technology (Jan. 1984) pp. 76-81.

ART-UNIT: 182
PRIMARY-EXAMINER: Draper; Garnette D.
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ATTY-AGENT-FIRM: Morrison & Foerster

ABSTRACT:

An improved method for expressing peptides as fusion proteins, uses a carrier for a heterologous peptide to provide a fusion protein having a high pI. The high isoelectric point facilitates separation of the fusion protein from all other host cell proteins, and separation of the carrier from the peptide after cleavage.

8 Claims, 13 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 10

Brief Summary Text - BSTX (12):

Shen et al, EP 163,406, disclosed a method for expressing small peptides as fusion proteins containing a leader protein and multiple copies of the peptide in tandem repeats. Shen used .beta.-galactosidase as the leader protein, and separated multiple copies of **insulin** with cleavable linker sequences (cleaving with trypsin and carboxypeptidase B). See also S. Cockle et al, "Protein Purification: Micro to Macro" (1987, Alan R. Liss, Inc) pp. 375-81.

Detailed Description Text - DETX (5):

The term "heterologous peptide" as used herein will generally refer to a peptide which is not endogenous to the host selected, although this definition will also include endogenous peptides in cases in which overexpression of such is desired. Heterologous peptides are short relative to most proteins, generally having a molecular weight of less

than about 10 kDa, and may be glycosylated, sialylated, phosphorylated, or the like. The peptide will also exhibit some form of useful activity, typically either biological activity (for example as a peptide hormone), or antigenic activity, for use in recombinant vaccines and/or immunological assays. The peptide will not include an accessible V8 cleavage site, so that the peptide is not fragmented during separation from the carrier protein. The peptide may either omit any cleavage site, or may express a site in an inaccessible portion of the peptide (e.g., at a position of the peptide which is masked by another portion of the peptide, or by gly-cosylation, phosphorylation, or the like). Peptide which naturally include a cleavage site for the selected protease may be altered, e.g., by site-specific mutagenesis, to a form in which the site is no longer present in cases where the activity of the peptide may be preserved. Representative peptides within the scope of the invention include, without limitation, atrial natriuretic peptide (ANP), brain natriuretic peptide, somatostatin, glucagon-like peptide, calcitonin, lung surfactant, **insulin,** growth hormone releasing factor (GRF), bradykinins, endorphins, enkephalins, and the like.

Detailed Description Text - DETX (33):

Second, double-stranded DNA from M13-hNF7 (Messing, supra) was prepared and digested with restriction endonucleases BamHI and BglII (New England Biolabs) and the approximately 700 bp DNA fragment containing the hANP* sequence was purified from a 0.6% agarose gel (Maniatis et al, supra, pp. 157-161). Plasmid pTrp-233 (prepared as described in U.S. Pat. No. 4,764,504) was digested with restriction endonuclease BamHI, its termini dephosphorylated by incubation with bacterial alkaline phosphatase (Amersham) by the method of Maniatis et al (supra, p. 133), and the linearized plasmid purified from a 0.6% agarose gel. Approximately 100 ng of the BamHI-BglII DNA fragment was mixed with 20 ng of the linearized plasmid and 400 U of T4 DNA ligase and ligation buffer added for overnight incubation at 15.degree. C. Competent E. coli MC1061 was transformed with the ligation and incubated overnight on L-plates with 100 .mu.g/mL ampicillin at 37.degree. C. Colonies were inoculated into L**-broth** containing 100 .mu.g/mL ampicillin, grown overnight, and 1 mL withdrawn to prepare plasmid DNA by the alkaline lysis method of Maniatis et al, (supra, p. 368-369). The orientation of the BamHI-BglII fragment within the plasmid was determined by digestion with restriction endonucleases PvuII and HindIII (New England Biolabs) and sizing of the DNA fragments on a 0.6% agarose gel. The desired orientation placed the BamHI recognition site of the fragment next to the EcoRI recognition site of the plasmid creating an hANP* cassette which could be released as an EcoRI-HindIII, EcoRI-BamHI, or EcoRI-EcoRI fragment. A plasmid with this orientation was designated phNF73.

Detailed Description Text - DETX (38):

First, 2 .mu.g of pTrp-233 were digested with EcoRI and the linearized plasmid purified from a 0.6% agarose gel. The termini were then filled in by the method of Maniatis et al (supra, p. 394), using the Klenow fragment of E. coli DNA polymerase I (Boehringer-Mannheim, Inc.). The mixture was then heated to 70.degree. C. for 5 min to inactivate the enzyme and T4 DNA ligase was added with ligation buffer for overnight incubation at 15.degree. C. E. coli MC1061 were made competent by the CaCl.sub.2 method and transformed as described by Maniatis et al (supra, pp. 250-251). Resulting ampicillin-resistant colonies were grown overnight in L **-broth** with 100 .mu.g/mL ampicillin and 1 mL aliquots withdrawn to prepare plasmid DNA by the alkaline lysis method of Maniatis et al (supra, pp. 368-369). A plasmid which had lost the EcoRI recognition site, as determined by failure to be digested by EcoRI, was designated pTRP81-6.

Detailed Description Text - DETX (57):

Fermentation

Detailed Description Text - DETX (58):

E. coli W3110 cells transformed with phNF117 were fermented by a fed-batch **fermentation** process in complex media containing an initial concentration of 5.0 g/L glucose. A concentrated glucose feed was started when the residual sugar was measured to be less than 0.5 g/L. The feed rate was then adjusted to maintain a residual glucose concentration of less than 1.0 g/L and a minimum dissolved oxygen of 20%. Fusion protein production was induced by the addition of 100 mg/L indole-acrylic acid (IAA) when the cell density was 40.0 OD.sub.590. The **fermentation** was continued for an additional 14.5 h and harvested at a cell density of 73.0 OD.sub.590. The total **fermentation** time was about 28.5 h.

Detailed Description Text - DETX (60):

Eighteen liters of **fermentation broth** were divided into six 1liter bottles and centrifuged at 5,000 rpm for 30 min at 4.degree. C. in a Sorvall RC-3B centrifuge. The supernatant was discarded, and the bottles refilled with whole **fermentation broth** and centrifuged again. This process was repeated three times, resulting in a recovery of 1.23 Kg cells (wet weight). All cells were frozen at -85.degree. C. until further processing. We have also used a cross-flow **microfiltration** unit equipped with 0.1 .mu.m **membranes** (Sartorius, Inc., Yauco, P.R.) to reduce the **fermentation broth** volume so that only one centrifugation step is required to recover the cells.

Detailed Description Text - DETX (72):

CM-Sepharose.RTM. purified fusion protein was shown to contain trace levels of E. coli proteolytic enzymes which led to product loss and nonspecific fragmentation of the fusion during prolonged incubation with immobilized Staph V8. These nonspecific proteases were inactivated by **heating** the CM-purified fusion protein to 80.degree. C. as follows. First, 0.1M Tris-SO.sub.4, pH 9.0, is heated to 80.degree. C. and mixed with an equal volume of purified fusion protein solution. The solution is maintained at 80.degree. C. for 30 min, then cooled to room temperature. During this treatment, the urea concentration is reduced to 3M so that it will not inhibit the cleavage reaction. If necessary, the volume of this solution may be reduced, prior to cleavage, by ultrafiltration with 10,000 MWCO **membranes**.

Detailed Description Text - DETX (73):

Staph V8 (endoproteinase Glu-C, Boehringer Mannheim GmbH, West Germany) was immobilized onto glutaraldehyde-activated PVC-silica composite **membranes** (Amerace, Inc., Hackettstown, N.J.) to a density of approximately 0.2 mg enzyme/cm.sup.2 **membrane**. (One may alternatively employ cyanogen bromide-activated Sepharose.RTM., or other activated resins, **membranes,** or substrates.) Three 47 mm diameter **membranes** containing approximately 15 mg Staph V8 were connected in parallel and equilibrated with 0.05M Tris-SO.sub.4, pH 9.0, at 2 mL/min at room temperature. Heat-treated fusion protein solution (200 mL) containing 1000 mg of protein was recirculated through the **membrane** system at 2 mL/min for 16 hr at room temperature. Cleavage was monitored by SDS-PAGE and RP-HPLC. FIG. 7A shows the RP-HPLC chromatogram of a sample prepared as described above, after purification on carboxymethyl Sepharose.RTM.. FIG. 7B shows the RP-HPLC chromatogram of the sample after cleavage with Staph V8. One may alternatively effect cleavage using soluble (not immobilized) enzyme.

Detailed Description Text - DETX (75):

After cleavage with immobilized Staph V8, ANP was separated from the high molecular weight cleavage products by simple size separation. Ultrafiltration of the cleavage mixture over an Amicon YM10 **membrane** (10,000 MWCO) with washing, provided about 80% recovery of the product in the filtrate stream, free of high molecular weight fragments.

Claims Text - CLTX (16):

6. The method of claim 5, wherein said peptide is selected from the group consisting of ANP, brain natriuretic peptide, somatostatin, glucagon-like peptide, calcitonin, lung surfactant, **insulin,** growth hormone releasing factor, bradykinins, endorphins, and enkephalins.

US-PAT-NO: 5417970

DOCUMENT-IDENTIFIER: US 5417970 A

****See image for Certificate of Correction****

TITLE: Drugs containing a glycosylated interleukin-2

DATE-ISSUED: May 23, 1995

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY	
Roskam, deceased; Willem	late of Montgiscard	N/A	N/A	FR	
Basuyaux; Bertrand	Courbevoie	N/A	N/A	FR	
Ferrara; Pascual	Villefranche de	N/A	N/A	FR	
Laporte; Martine	Lauragais	N/A	N/A	FR	
Maureaud; Thierry	Ramonville Saint-Agne	N/A	N/A	FR	
Vita; Natalio	Auzielle	N/A	N/A	FR	
Bayol; Alain	Toulouse	N/A	N/A	FR	
Perry; Genevieve	Tournefeuille	N/A	N/A	FR	Toulouse

ASSIGNEE INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY	TYPE CODE
Sanofi	Paris	N/A	N/A	FR	03

APPL-NO: 08/152886

DATE FILED: November 16, 1993

PARENT-CASE:

This application is a continuation of application Ser. No. 07/715,862, filed Jun. 17, 1991, which in turn is a continuation of Ser. No. 07/499,472, filed on Jun. 21, 1990, both now abandoned.

FOREIGN-APPL-PRIORITY-DATA:

COUNTRY	APPL-NO	APPL-DATE
FR	88 13865	October 21, 1988
FR	89 05150	October 21, 1988

INT-CL-ISSUED: [06] A61K037/02 , C07K003/20 , C07K003/22 , C07K015/14

INT-CL-CURRENT:

TYPE	IPC DATE
CIPS	C07 K 14/435 20060101
CIPS	C07 K 14/55 20060101
CIPS	C12 P 21/02 20060101
CIPN	A61 K 38/00 20060101

US-CL-ISSUED: 424/85.2 , 435/69.52 , 530/351 , 530/416 , 530/417

US-CL-CURRENT: 424/85.2, 435/69.52 , 530/351 , 530/416 , 530/417

FIELD-OF-CLASSIFICATION-SEARCH: 530/351; 530/412 ; 530/416 ; 530/417 ; 435/69.12 ; 435/71.1 ; 435/72.85 ; 424/85.2 ; 514/12 ; 514/801

****See application file for complete search history****

REF-CITED:

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4675383	June 1987	Bohlen et al.	520/351	N/A	N/A
4778879	October 1988	Mertelsmann et al.	530/351	N/A	N/A
5217881	June 1993	Park	436/546	N/A	N/A

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ART-UNIT: 185
PRIMARY-EXAMINER: Russel; Jeffrey E.
ATTY-AGENT-FIRM: Foley & Lardner

ABSTRACT:

An interleukin-2 preparation suitable for pharmaceutical purposes, consisting essentially of disialilated glycosylated interleukin-2, monosialilated glycosylated interleukin-2, or a mixture thereof, and substantially free of organic solvents, is isolated from recombinant CHO cells transformed with a vector containing a DNA sequence coding for a natural precursor of human interleukin-2, and is purified by a multi-step process.

25 Claims, 9 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 9

Brief Summary Text - BSTX (22):

Advantageously the fraction rich in interleukin.sub.-- 2 is separated from the culture supernatant by double **filtration** between a **membrane** 1 permeable to the protein and a **membrane** 2 which retains it. **Membrane** 1 is e.g. a **microfiltration** or ultrafiltration **membrane**. **Membrane** 2 is an ultrafiltration **membrane**. Preferably the **membrane** 1

has a stop threshold above 30 kDa, more particularly between 50 and 150 kDa, and **membrane** 2 has a stop threshold below 10 kDa, preferably between 5 and 10 kDa. **Membranes** 1 and 2 are advantageously made of cellulose acetate and polysulphone.

Detailed Description Text - DETX (47):

f) The **temperature** of the culture was adjusted to about 37.degree. C.;

Detailed Description Text - DETX (50):

The fermentation process was in two parts, called the growth phase and the production phase. During the growth phase, foetal calf serum (5%) was added to the medium. During the production phase, the medium was mixed with a protein fraction called F IV-1, isolated from bovine serum by the Cohn fractionation method. The culture medium was moderately rich in proteins (400 mg of total proteins/l). Production was continued for at least 30 days. The harvest was cooled in line at a **temperature** of +6.degree. C. The concentration of total proteins was 500 mg/l. The CTLL-2 activity in the culture supernatant was 40,000 U/ml, i.e. an IL-2 concentration of about 2 mg/l (assuming the IL-2 specific activity to be 2.times.10.sup.7 U/mg and the IL-2 protein purity to be 0.4%). This culture supernatant will hereinafter be called culture supernatant A.

Detailed Description Text - DETX (58):

f) **Temperature** of culture adjusted to about 37.degree. C., and

Detailed Description Text - DETX (60):

The **fermentation** process was in two parts, called the growth phase and the production phase. During the growth phase the medium was mixed with foetal calf serum (2.5%). During the production phase, the serum was replaced by 3 mg/l of **insulin** and 1 mg/l of lactoferrin, the only proteins in the medium.

Detailed Description Text - DETX (61):

During this phase, 0.5% of polyvinylpyrrolidone having an average molecular weight of 40,000 was also added to the medium. It has been found that this polymer can increase the specific output of IL-2 from recombined CHO cells (the quantity of IL-2 secreted per unit time and unit biomass). Accordingly, the culture medium was a synthetic medium low in proteins (4 mg/l of total proteins). Production was kept up for at least 30 days. The harvest was cooled in line at a **temperature** of +6.degree. C. The concentration of total proteins in the culture supernatant was 100 mg/l. The CTLL-2 activity was 120,000 U/ml, i.e. a concentration of about 6 mg/l and a protein purity of 6%. The culture supernatant will hereinafter be called culture supernatant B.

Detailed Description Text - DETX (79):

130 liters of the supernatant of medium B described in Example 3 were taken and pre-filtered on an 8 .mu.m stop threshold filter so as to eliminate large particles capable of clogging the ultrafiltration **membranes**.

Detailed Description Text - DETX (80):

The interleukin.sub.-- 2, the molecular weight of which was between 15 kDa and 17 kDa, was fractionated and then concentrated by double tangential ultrafiltration between a first **membrane** having a stop threshold of 100 kDa and a second **membrane** having a stop threshold of 10 kDa, operating in the manner described hereinafter. The first and the second **membrane** were spiral cartridges of cellulose acetate, i.e. **membranes** YM 100 and YM 10 said by Messrs AMICON, mounted in cascade so that the filtrate from the first **membrane** supplied the material retained by the second **membrane**.

Detailed Description Text - DETX (81):

The prefiltrate was fed to the first **membrane** and, to begin with, the material retained by the first **membrane** and by the second **membrane** was concentrated until the volume of material retained by the first **membrane** was about 15 liters. Next, the retained material was diafiltered (washed at constant volume) with 80 liters of ultrapurified water so as to remove all the IL-2 from the material retained by the first **membrane** and reduce the ionic force of the material retained by the second **membrane**. Next, the latter material was concentrated to 1.5 liters and the interleukin.sub.-- 2 was recovered after rinsing the **membrane** with water. The resulting concentrate was filtered through a filter having an 0.2 .mu.m stop threshold, so as to eliminate certain precipitates formed during ultrafiltration. The product was 2.4 liters of concentrated aqueous solution of IL-2.

Detailed Description Text - DETX (97):

After the solution obtained at the end of step c) had been concentrated on a cellulose acetate spiral **membrane** having a stop threshold of 10 kDa, the solution was injected into the column, which had previously been balanced with a 50 mM aqueous solution of sodium phosphate at pH 6.5, the glycosylated IL-2 was then eluted with the last-mentioned solution. The solution was detected at the column outlet by measuring the optical density at 280 nm. The various fractions collected were combined in accordance with their purity, analysed by reverse phase HPLC.

Detailed Description Text - DETX (120):

The product, reduced and carboxymethylated, was digested overnight at ambient **temperature** by the action of trypsin added in a proportion of 1/30 (weight/weight) and

the peptides obtained were separated by reverse phase HPLC chromatography by means of an acetonitrile gradient (in the presence of 0.1% trifluoroacetic acid) by the method published in Journal of IKmmunological Methods (1985), 81, 15-30.

Detailed Description Text - DETX (136):

200 liters of supernatant of culture A described in Example 3 were taken, separated, and a fraction rich in IL-2 was concentrated by double ultrafiltration between a first polysulphone **membrane** having a 100 kDa stop threshold and a second polysulphone **membrane** having a 10 kDa stop threshold. The operation was performed in the same manner as in Example 5 hereinbefore. The product was 5.6 liters of concentrated aqueous solution of IL-2.

Detailed Description Text - DETX (147):

The solution obtained at the end of step b) was concentrated on a cellulose acetate **membrane,** i.e. the YM 10 **membrane** sold by Messrs AMICON and having a stop threshold of 10 kDa. The solution was then injected into the column, which had first been balanced with an aqueous solution (pH 6.5, molarity 0.1M) of sodium phosphate. The glycosylated IL-2 was then eluted with the last-mentioned solution.

Detailed Description Text - DETX (206):

Temperature 22.degree. C. (.+-.2.degree. C.)

Detailed Description Text - DETX (254):

Temperature: 22.degree. C. (.+-.2.degree. C.)

Detailed Description Text - DETX (317):

Experimental freeze-drying of an aqueous solution of sodium phosphate, pH 6.5, containing glycosylated IL-2 obtained as described in Example 5 were made in the presence or absence of various excipients, present in variable proportions. The solute was then reconstituted, its appearance was observed, its pH was measured, and the quantity of glycosylated IL-2 present, was determined by HPLC on a reverse phase column (compare Example 5-3) and the CTLL-2 activity was measured. These operations were made immediately after freeze-drying after the substance had been preserved for one year at a **temperature** of 4.degree. C., and after preservation for three months at a **temperature** of 25.degree. C. and after preservation for three months at a **temperature** of 37.degree. C.

Detailed Description Text - DETX (325):

The foregoing results show that, after freeze-drying, glycosylated IL-2 has excellent stability for acceptable pharmaceutical formulations. Probably the freeze-dried material can be preserved for several years at a **temperature** of 4.degree. C. It is also found that in all cases the reconstituted solute is clear and at a physiological pH without adding a toxic agent or chemically modifying the molecule to make it soluble. These properties of glycosylated IL-2 differ from E. coli derived IL-2. The latter substance loses more than 50% of its activity and gives opalescent solutions after freeze-drying (see European Patent Application No. 0 158 487, page 10). To soluble therefore, it is necessary to add toxic solubilising agents such as SDS (Sodium Dodecyl Sulphate).

Claims Text - CLTX (6):

6. An interleukin-2 preparation according to claim 1, characterised in that the glycosylated interleukin-2 retains its initial CTLL-2 activity after lyophilisation in an aqueous solution of pH 6.5 to which hydrolysed gelatin or human serum albumin has been added, preservation of the lyophilised product at a **temperature** of 4.degree. C. for one year, and reconstitution of the solute.

Claims Text - CLTX (7):

7. An interleukin-2 according to claim 1, characterised in that the glycosylated interleukin-2 retains its initial CTLL-2 activity after lyophilisation in an aqueous solution of pH 6.5 to which hydrolysed gelatin and alanine have been added, preservation of the lyophilised product at a **temperature** of 25 .degree. C. for 3 months, and reconstitution of the solute.

Claims Text - CLTX (8):

8. An interleukin-2 preparation according to claim 1, characterised in that the glycosylated interleukin-2 retains its initial CTLL-2 activity after lyophilisation in art aqueous solution of pH 6.5 to which hydrolysed gelatin and alanine have been added, preservation of the lyophilised product at a **temperature** or 37.degree. C. for 3 months, and reconstitution of the solute.

Claims Text - CLTX (21):

17. An interleukin-2 preparation according to claim 13, wherein step (a) of said process comprises filtering a supernatant from said cell culture between a first **membrane** having a stop threshold between 30-150 kDa, and filtering said supernatant between a second **membrane** having a stop threshold between 5-10 kDa, and collecting a fraction rich in interleukin-2 and glycosylated interleukin-2.

US-PAT-NO: 6531448

DOCUMENT-IDENTIFIER: US 6531448 B1

****See image for Certificate of Correction****

TITLE: Insoluble compositions for controlling blood glucose

DATE-ISSUED: March 11, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Brader; Mark Laurence	Indianapolis	IN	N/A	N/A

ASSIGNEE INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY	TYPE CODE
Eli Lilly and Company	Indianapolis	IN	N/A	N/A	02

APPL-NO: 09/217275

DATE FILED: December 21, 1998

PARENT-CASE:

This Application claims benefit to Ser. No. 60/068,601 (Dec. 23, 1997) Ser. No. 60/088,859 (Jun. 11, 1998) Ser. No. 60/109,940 (Nov. 25, 1998)

INT-CL-ISSUED: [07] A61K038/28 , C07K007/00

INT-CL-CURRENT:

TYPE	IPC DATE
CIPP	A61 K 38/28 20060101

US-CL-ISSUED:	514/3 , 514/4 , 514/314 , 424/491 , 530/303 , 530/304 , 530/345
US-CL-CURRENT:	514/3, 424/491 , 514/314 , 514/4 , 530/303 , 530/304 , 530/345
FIELD-OF-CLASSIFICATION-SEARCH:	530/303; 530/304 ; 530/345 ; 514/314 ; 514/317 ; 424/491

****See application file for complete search history****

REF-CITED:

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FOREIGN-PAT-NO	PUBN-DATE	COUNTRY	US-CL
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Claims for U.S. Application No. 09/761,903 (Claims 110-162).

Claims for U.S. Application No. 10/010,038 (Claims 85-118).

ART-UNIT: 1654